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Annual subscription £30.00 (£35.50 USA and Canada). (Payment may be made in any currency at the current exchange rate. Orders must be accompanied by remittance. Prices applicable only to orders received before 31st December 1975.)

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Advances in Drug Research

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December 1974, x + 348pp., £9.80/$26.00 0.12.013308.3

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David Parkes (*King's College Hospital and the Maudsley Hospital, London, England*): **Amantadine.**
Alan Bennett (*King's College Hospital Medical School, London, England*): **Prostaglandin antagonists.**
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E. M. Vaughan Williams (*The University of Oxford, England*): **The electrophysiological basis for a rational approach to antidysrhythmic therapy.**
J. R. Parratt (*University of Strathclyde, Glasgow, Scotland*): **Pharmacological approaches to the therapy of angina.**
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Systematic Pharmacology

SP1	Cardiovascular system
SP2	Central nervous system
SP3	Other physiological systems

Pharmacokinetics

PK1	Storage, distribution, fate
PK2	Synthesis, metabolism
PK3	Uptake, release

Autopharmacology

AP1	Adrenergic, cholinergic transmission
AP2	Histamine, kinins, prostaglandins, etc.
AP3	Hormones

Drug Mechanisms

DM1	Molecular, cellular, biochemical, biophysical
DM2	Receptors, antagonism, potentiation, tolerance

CP	**Clinical Pharmacology, Experimental Therapy**
CT	**Chemotherapy**
M	**Miscellaneous**
PM	**Pharmacometrics**
PP	**Psychopharmacology**
TX	**Toxicology**

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LOCAL ANAESTHETIC AND ANTIARRHYTHMIC PROPERTIES OF AN AMINOSTEROID: 3α-DIMETHYLAMINO-5α-ANDROSTAN-2β-OL-17-ONE (ORG. NA13)

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Organon Laboratories Ltd, Newhouse, Lanarkshire ML1 5SH, Scotland

B.B. VARGAFTIG[2]
Organon S.a.r.l., 60146-Eragny-sur-Epte, France

1 The aminosteroid Org. NA13 (3α-dimethylamino-5α-androstan-2β-ol-17-one) was shown to be a more potent local anaesthetic than lignocaine in rats and guinea-pigs.

2 Experimental arrhythmias induced in mice by chloroform, in rats by aconitine and in dogs by coronary artery ligation were corrected by Org. NA13 at doses from 10 to 50 mg/kg intravenously.

3 In contrast to lignocaine, other local anaesthetics and β-adrenoceptor blocking drugs, Org. NA13 did not show any activity against the arrhythmias induced by ouabain in dogs.

4 The acute toxicity in whole animals and myocardial toxicity in the rabbit isolated atrium appeared to be less than that observed with lignocaine.

Introduction

Recent advances in the pharmacology of steroids have led to the clinical introduction of therapeutic agents possessing pharmacodynamic properties other than those of an endocrinological or related nature. In particular the class of steroids possessing extranuclear amino groups, known as aminosteroids, has yielded many different types of activity (Buckett, 1972). For example, potent neuromuscular blocking agents such as pancuronium bromide (Pavulon) (Buckett, Marjoribanks, Marwick & Morton, 1968), analgesics (Craig, 1968), central nervous system depressants (Hewett, Savage, Lewis & Sugrue, 1964) and local anaesthetics (Buckett, Hewett, Marwick & Savage, 1967; Hewett & Savage, 1968) have all been reported.

This paper describes the properties of Org. NA13, 3α-dimethylamino-5α-androstan-2β-ol-17-one (Figure 1), a potent local anaesthetic which was selected for further pharmacological study from the series described by Buckett *et al.* (1967). The evidence for its local anaesthetic and anti-arrhythmic activity is presented in comparison with the standard drug, lignocaine hydrochloride.

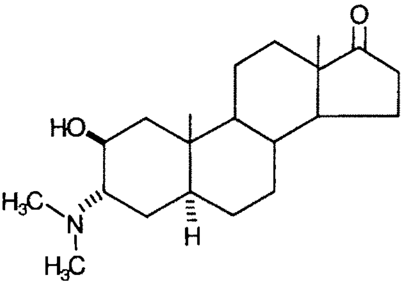

Figure 1 The chemical structure of 3α-dimethylamino-5α-androstan-2β-ol-17-one (Org. NA13).

Methods

Local anaesthesia

Intracutaneous local anaesthetic potency was determined by the method of Bülbring & Wajda (1945) using groups of six female albino guinea-pigs (600-700 g body wt.). For each experiment three concentrations of test and standard drug were injected intracutaneously into six injection sites. The flinch response to a needle prick on each site at 6 min intervals up to 36 min was recorded and converted to a percentage for each concentration, allowing a potency ratio to be determined.

---

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Surface anaesthetic potency was determined in guinea-pigs (600 g body wt.) according to the method of Chance & Lobstein (1944). Drugs were dissolved in McIlwain buffer (pH 6.0) and solutions instilled into the open eye for 15 seconds. The corneal reflex was tested by touching the eye surface with a glass rod at 1 min intervals for 10 minutes. Potency ratios were determined from dose-response lines obtained from three concentrations of each drug.

Conduction anaesthesia was estimated by rat sciatic nerve block according to the method of Åstrom & Persson (1961) using groups of six male albino Wistar rats (150-200 g body wt.).

Anti-arrhythmic actions

Prevention of chloroform-induced arrhythmias was examined in male albino mice (20-24 g) by the method of Lawson (1968).

Delay in onset of aconitine-induced ventricular arrhythmias was examined in male albino Wistar rats (350-400 g body wt.) anaesthetized with pentobarbitone sodium (60 mg/kg i.p.). Animals were pretreated with NA13, lignocaine or 0.9% w/v NaCl solution (saline) intravenously 2 min before infusion of aconitine 10 µg/ml by the intravenous route according to the method described by Vargaftig & Coignet (1969).

Changes in auricular refractory period were measured essentially by the method of Dawes (1946) using isolated atria of rabbits. Atria were suspended by stainless steel stimulating electrodes in a 50 ml isolated organ bath containing Ringer-Locke solution at 30°C equilibrated with 95% O_2 and 5% CO_2. Contractions were recorded via a force displacement transducer (Statham FT03) on a polygraph (Grass 7) from which atrial rate and amplitude of contraction could be measured directly. The spontaneously beating preparation was allowed a stabilization period of 1 h before stimulation by trains of impulses of increasing frequency for 20 s in each minute using a Grass S4 stimulator. The first frequency at which atria did not respond 1 : 1 to stimulation was taken as the reciprocal of the functional refractory period. For determining drug effects, the test anti-arrhythmic drug was added in solution to the bath for 10 min prior to stimulation, during which time direct effects on the spontaneously beating atria could also be observed.

Antagonism of ouabain intoxication in pentobarbitone anaesthetized dogs was studied by infusion of antiarrhythmic drugs at 1 mg kg^{-1} min^{-1} intravenously for 10 min after injections of ouabain The intoxication was produced by intravenous ouabain at 0.04 mg/kg followed 30 min later by 0.02 mg/kg and then at 10 min intervals by 0.01 mg/kg until ventricular tachycardia was observed on the electrocardiogram which was then continuously monitored throughout the infusion of anti-arrhythmic drug and for 30 min thereafter.

Correction of post-infarction arrhythmias was demonstrated in six male dogs (8-10.3 kg body wt.) prepared essentially according to the metho of Harris (1950) except that a four stage ligatio procedure for the anterior descending branch o the left coronary artery was used to reduce the mortality experienced after the two-stage method. Five to 10 h post-operatively the initial sinus rhythm was replaced by ventricular tachycardia. The anti-arrhythmic drugs were infused over a period of 10 min to unanaesthetized dogs 20-40 h after surgical preparation and electrocardiogram recorded from three derivations (Alvar Reega VIII).

Toxicity

Acute toxicity of anti-arrhythmic drugs in mic was determined after intravenous, subcutaneou intraperitoneal and oral administration to albin male mice (18-22 g) with LD_{50} values and 95 limits being calculated by standard methods.

Mucosal irritation was examined by applying drug solutions to the right eyes of groups of three New Zealand white rabbits at a concentration of 1% in normal saline. Saline was instilled into the left eyes. The animals were observed at 1, 12 and 24 h for presence or absence of irritation to the eye and conjunctiva.

Drugs

The following drugs were used: lignocaine hydrochloride (MacFarlan Smith Ltd; Laboratoires Roger Bellon), ouabain, acetylcholine chloride and aconitine (Sigma), which was dissolved in distille water acidified with HCl to effect solution. Org. NA13 (3α-dimethylamino-5α-androstan-2β-ol-17-one) was kindly made available as the water soluble hydrochloride salt by Dr D.S. Savage, Organon Laboratories Ltd. All doses and result are expressed in terms of salt.

Results

Local anaesthetic activity

The aminosteroid NA13 was shown to possess more potent local anaesthetic properties than lignocaine in guinea-pigs. After intracutaneous application the effective concentration of NA13 to produce anaesthesia in 50% of test sites was 0.67 (s.e. mean 0.08) mg/ml, in contrast to lignocaine which had an effective concentration of 1.73 (s.e. mean 0.21) mg/ml indicating a potency ratio of 2.6 for NA13 (lignocaine = 1.0) in three determinations in guinea-pigs. As a surface anaesthetic NA13 was also more potent than lignocaine. In three determinations the effective concentration of NA13 to produce anaesthesia on 50% of sites was 1.33 (s.e. mean 0.23) mg/ml, whereas that for lignocaine was 2.55 (s.e. mean 0.47) mg/ml, from which a potency ratio of 1.92 was calculated for NA13 (lignocaine = 1.0).

The conduction blockade as demonstrated in the rat sciatic-nerve *in vivo* was similar for both NA13 and lignocaine (Table 1). The onset of the blockade was less rapid after NA13. At the higher concentration NA13 exhibited a longer duration of action than lignocaine, but the duration at lower concentrations was similar or shorter.

Anti-arrhythmic activity

Mice Against chloroform-induced ventricular arrhythmias both NA13 and lignocaine were effective after intraperitoneal injection in mice The ED_{50} value for NA13 was 46 (39-52) mg/kg and for lignocaine 49 (43-53) mg/kg.

Rats NA13 was highly effective in delaying the onset of the first burst of ventricular arrhythmias induced by a slow intravenous infusion of aconitine to the anaesthetized animal. Table 2 illustrates the increase in aconitine tolerated after pretreatment with NA13. Significant increases are evident following doses of 25 and 50 mg/kg NA13 intravenously. Similarly lignocaine had a significant effect at 25 mg/kg, but increasing the dose to

Table 1 The activity of NA13 and lignocaine in producing sciatic nerve block in rats

Compound	*Concentration of drug (%)*	*Onset of local anaesthesia (min)*	*Duration of local anaesthesia (min ± s.e.)*
NA13	2	7	98 ± 6.3
NA13	1	5	65 ± 6.6
NA13	0.5	5	22 ± 0.3
Lignocaine	2	5	63 ± 6.6
Lignocaine	1	3	56 ± 4.2
Lignocaine	0.5	3	37 ± 3.5

Results from two series of experiments in which each animal received NA13 on one side and lignocaine on the other. Method of Åstrom & Persson (1961).

Table 2 The amount of aconitine by intravenous infusion required to induce ventricular arrhythmias in rats pretreated intravenously with NA13 and lignocaine

Drug	*Dose (mg/kg i.v.)*	*Number of animals*	*Aconitine tolerated (µg/kg ± s.e.)*	*% Increase in aconitine*	*P Value*
Control	—	14	32.3 ± 2.5	—	—
Lignocaine	6.25	3	35.7 ± 4.4	11	NS
	12.5	5	48.6 ± 15.6	20	NS
	25	5	50.9 ± 9.1	58	0.05
	50*	3	—	—	—
NA13	6.25	3	43.1 ± 7.5	33	NS
	12.5	3	59.1 ± 16.3	82	NS
	25	4	71.5 ± 6.7	121	0.05
	50	3	73.9 ± 9.3	128	0.05

* Toxic dose level.

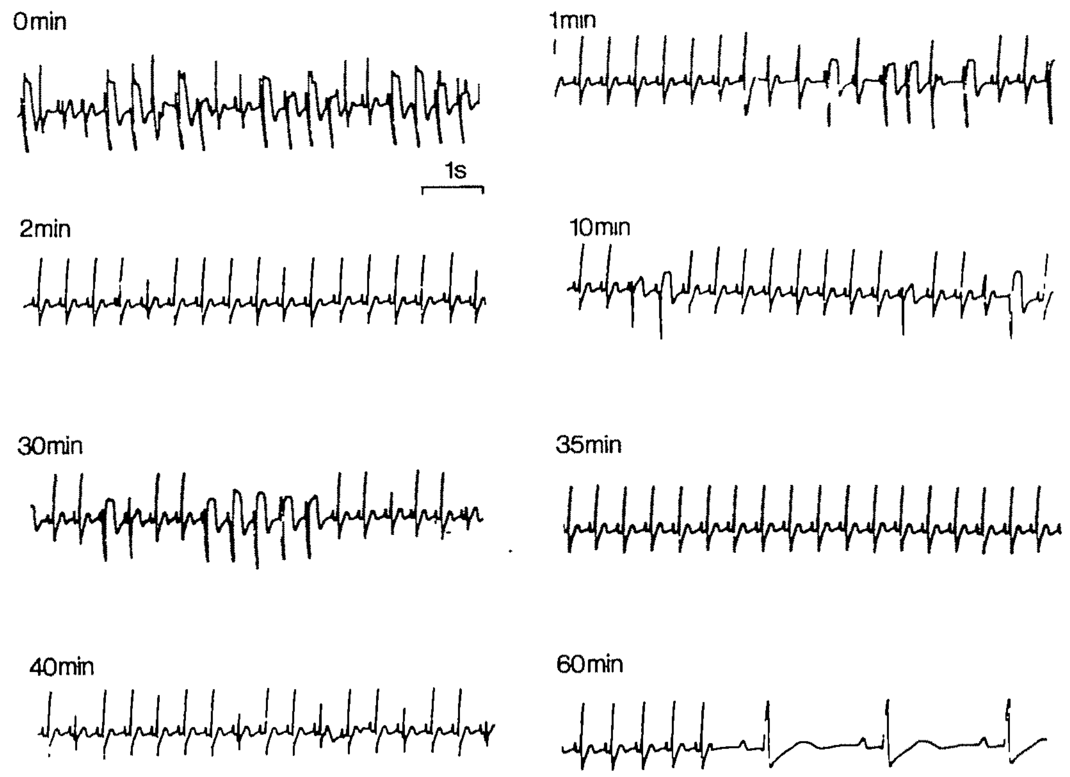

Figure 2 The action of NA13 in correcting the ventricular arrhythmia in a dog (No. 2) 20 h after coronary artery ligation (electrocardiogram, lead II). The figure above the record indicates time in min after the start of NA13 infusion 1 mg kg^{-1} min^{-1} for 0-10 minutes. At 30 min the infusion was repeated. Time scale = 1 second.

50 mg/kg was toxic in all animals. It is clear that NA13 is somewhat more active and less toxic than lignocaine in this test.

Rabbits The examination of the drugs on isolated atria of rabbits demonstrated the considerable increase in refractory period shown in Table 3. On a molar basis lignocaine was four times as active as NA13 in contrast to the results described above for local anaesthetic and anti-arrhythmic actions in rats and mice. Nevertheless evidence from Table 3 also suggests that lignocaine

Table 3 The effect of NA13 and lignocaine on the functional refractory period and on the contractions of spontaneously beating rabbit atria *in vitro*

Drug	*Concentration ($M \times 10^{-5}$)*	*Number of experiments*	*% Increase in refractory period (± s.e.)*	*Onset to 50% decrease in amplitude of contraction (min)*	*% Reduction in atrial beat frequency (± s.e.)*
NA13	1.65	6	13.0 ± 4.1		
NA13	3.3	6	18.4 ± 7.7	–	8.13 ± 0.6
NA13	6.6	6	43.4 ± 4.3	10	27.1 ± 13.0
NA13	13.2	6		6	23.4 ± 5.2
Lignocaine	0.42	5	21.1 ± 2.8		
Lignocaine	0.83	5	43.0 ± 10.4		
Lignocaine	1.65	5	43.5 ± 6.6		
Lignocaine	3.3	5		1.5	0
Lignocaine	6.6	5		1	0
Lignocaine	13.2	5		<1	100

Table 4 Effects of NA13 and lignocaine on the ventricular arrhythmia induced by artery ligation in dogs

		Pre-drug heart rate		*Heart rate after start of drug administration*											
				0-1 min		*1-5 min*		*5-10 min*		*10-15 min*		*15-20 min*		*20-30 min*	
Dog No.	*Drug*	*V.R.*	*% Sinusal*	*V.R.*	*% S*	*V.R.*	*% S*	*V.R.*	*% S*	*V.R.*	*% S*	*V.R.*	*% S*	*V.R.*	*% S*
1	NA13	171	0	168	3	161	87	180	100	180	50	168	50	180	30
2	NA13	135	27	132	69	135	74	135	88	136	100	136	100	135	40
2a	NA13	135	40	135	45	126	88	135	100	128	100	128	100	126	100
3	NA13	144	0	144	100	144	87	144	44	135	100	144	81	162	0
4	Lignocaine	234	0	225	4	216	4	180	0	180	10	189	9	180	23
5	Lignocaine	144	0	156	0	156	0	162	0	160	0	160	0	158	0
6	Lignocaine	162	0	156	0	156	0	156	100	144	100	144	91	138	91

The ventricular rate (V.R.) and percentage of sinus beats (% S) are given for before, during (0-10 min) and after (10-30 min) Intravenous Infusion of 1 mg kg^{-1} min^{-1} (total dose· 10 mg/kg) of NA13 and of lignocaine in dogs displaying ventricular arrhythmias due to coronary ligation 20-40 h previously.

may be more depressant on the myocardium when its effect on the amplitude of contraction of spontaneously beating atria is considered. For lignocaine the decrease to 50% of contraction occurred at similar times with only one tenth of the molar concentration of NA13 which was required for this effect NA13 itself caused a small reduction in the rate of contraction at these concentrations.

Dogs NA13 in doses up to 50 mg/kg infused intravenously was completely ineffective in reverting ouabain-induced ventricular tachycardia to sinus rhythm in four anaesthetized dogs. In contrast lignocaine administered at 1 mg/kg increased the percentage of sinus beats from 5 ± 3.5 (±s.e.) before to 25 ± 14 immediately after intravenous infusion (four dogs). At 2 mg/kg lignocaine increased the percentage from 3 ± 2.9 to 79 ± 20 after infusion (four dogs).

Seven observations were carried out in six dogs 20-40 h after ligation of the anterior descending branch of the left coronary artery. NA13, administered by a slow intravenous infusion of 1 mg kg^{-1} min^{-1} over 10 min reverted the ventricular tachycardia (animals 1 and 3) or the polymorphous ventricular extrasystoles (animal 2) to sinus rhythm as shown in Table 4. The ECG from animal 2 is shown in Figure 2 which clearly demonstrates the corrective effect of NA13. In this case a second perfusion was carried out to effect full correction at 60 minutes.

Lignocaine at this dose level showed correction in one of the three dogs tested, whereas no corrective action on the remaining two dogs was observed possibly due to rapid metabolism of this compound after intravenous administration.

Toxicity

By intravenous and intraperitoneal routes in mice, NA13 was less toxic than lignocaine (Table 5). It was more toxic after subcutaneous administration. Both drugs exhibited similar toxic signs namely, tremor, excitation and convulsions. Neither compound showed toxicity after oral administration. After application of NA13 solutions to the rabbit eye, no mucosal irritation was observed up to 24 h later. Similarly the guinea-pigs used in the surface anaesthesia experiments did not show any evidence of irritation.

Discussion

Org. NA13 has been shown to be a potent local anaesthetic compound in three types of test designed to show conduction anaesthesia, topical or surface anaesthesia and infiltration anaesthesia

Table 5 The acute toxicity of NA13 and lignocaine by various routes in mice

Drug	*Route*	*LD_{50} mg/kg (95% limits of error)*
NA13	Intravenous	56.5 (37.5-71.2)
NA13	Intraperitoneal	156 (127-195)
NA13	Subcutaneous	201 (153-262)
NA13	Oral	>300
Lignocaine	Intravenous	28.5 (18.7-43 0)
Lignocaine	Intraperitoneal	117 (115-119)
Lignocaine	Subcutaneous	>300
Lignocaine	Oral	>300

after intracutaneous injection. The local anaesthetic properties of steroids have only previously been noted in steroidal alkaloids such as conessine, isoconessine and neoconessine (Stephenson, 1948). With these compounds local anaesthesia was found after intracutaneous injection in guinea-pigs, but the irritation after injection as well as undesirable pharmacological properties such as neuromuscular blockade and myocardial depression precluded any further interest.

Chemically NA13 bears little structural resemblance to the known local anaesthetics, nevertheless it possesses a large lipophilic nucleus and two hydrophilic groups at the 2 and 3 positions. These substitutions are probably optimal since analogues of NA13 do not show greater potency as local anaesthetic drugs (Buckett, 1972). The mode of action of NA13 has been confirmed in experiments with frog sciatic nerve *in vitro*, when the action potentials initiated by stimulation were blocked in passage along the nerve by solutions of the compound (Duff & Nicol, personal communication).

The increasing interest in the use of local anaesthetic drugs to control arrhythmias led us to study the anti-arrhythmic properties of NA13 in some detail. In the initial tests NA13 showed similar activity to lignocaine in chloroform-induced arrhythmias in mice and would thus be expected to be more active than procainamide and quinidine (Lawson, 1968). The action of NA13 in rats was clearly preferable to lignocaine, since the former compound produced anti-arrhythmic actions at doses below toxic levels whereas the standard drug was toxic at therapeutic dose levels against aconitine in this species. The results from experiments on the rabbit isolated atria also suggest some differences in the activity of the two compounds, with lignocaine being apparently more cardiotoxic, so it is clearly important to have confirmation of such actions in more than one species before clinical assessment of a new drug of this type.

NA13 also shows potent anti-arrhythmic actions in coronary artery ligated dogs but is unexpectedly inactive up to 50 mg/kg against ouabain-induced ventricular arrhythmias in the same species. This inactivity was also found in cats (Buckett, unpublished observations). However, our findings with lignocaine on ouabain arrhythmias are in agreement with Allen, Shanks & Zaidi (1971) who found a dose of 0.9 mg/kg of lignocaine intravenously produced a 50% reduction in the number of ventricular ectopic beats. The activity of other drugs possessing local anaesthetic properties, such as procaine, procainamide and quinidine has also been demonstrated against ouabain arrhythmias in dogs (Mosey & Tyler, 1954) so the profile of NA13 is indeed unusual. It is free from β-adrenoceptor blocking actions (Vargaftig, unpublished observations) so cannot be compared with propranolol which is active against ouabain-induced arrhythmias in the dog (Allen *et al*, 1971) at low doses and whose activity is due partially, at least, to its local anaesthetic action (Barrett & Cullum, 1968).

In these experiments NA13 showed activity in correcting post-infarction arrhythmias to sinus rhythm in all dogs tested, whereas lignocaine was only effective under these conditions in one out of three dogs. This may well be due to the inactivation of lignocaine, since it has been emphasized (Allen *et al*, 1971) that the rapid metabolism is responsible for the variability in its use and difficulty in using it as a standard. It is unlikely that NA13 is so rapidly metabolized and indirect evidence for this is given by the LD_{50} values of the two drugs. Lignocaine and NA13 show LD_{50} values of the same order after rapid intravenous administration, where there is no time for metabolic transformation, whereas after subcutaneous administration allowing metabolism then lignocaine is much less toxic than the aminosteroid. Foldes (1966) used the intravenous LD_{50} in mice as a guide to clinical potency and the subcutaneous LD_{50} as an indication of metabolic transformation of local anaesthetic drugs.

Our observations indicate that NA13 is a novel steroidal local anaesthetic drug possessing a profile of anti-arrhythmic action, inactive against ouabain-induced arrhythmias, a property which we are unable to explain at the present time.

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(Received November 8, 1974.
Revised January 4, 1975.)

Br. J. Pharmac. (1975), 54, 11-15

ADRENOCEPTORS IN INTRACEREBRAL RESISTANCE VESSELS

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1 The effects of tyramine and isoprenaline on hypothalamic blood flow (HBF) were measured in conscious rabbits.

2 Injections of small doses of tyramine caused an increase in HBF while larger doses caused a decrease in HBF.

3 Isoprenaline injections also produced an increase in HBF.

4 The vasodilatation induced by isoprenaline and the small dose of tyramine was blocked by propranolol.

5 The vasoconstriction induced by the larger doses of tyramine was abolished by phenoxybenzamine.

6 Chemical sympathectomy of the hypothalamus with 6-hydroxydopamine and depletion of biogenic amines by reserpine also abolished tyramine-induced vasoconstriction.

7 These results suggest the presence of α- and β-adrenoceptors in cerebral resistance vessels, and that these receptors may be activated by released (endogenous) noradrenaline.

Introduction

The functional importance of the cerebrovascular nerves is a subject of considerable controversy. Early observations around the turn of the century (Bayliss, Hill & Gulland, 1895; Hill & MacLeod, 1901) suggested that these vasomotor nerves were of minor importance in the regulation of cerebral blood flow. This view is still widely held.

Removal of the superior cervical ganglion appears to have little effect on total cerebral blood flow (Shackleford & Hegedus, 1966; Waltz, Yamaguchi & Regli, 1971a), or on the ability of the cerebral vasculature to autoregulate (Eklöf, Ingvar, Kagström & Olin, 1971) or respond to CO_2 (Waltz, Yamaguchi & Regli, 1971b). On the other hand, James, Millar & Purves (1969) have demonstrated a decrease in cerebral blood flow following stimulation of the cervical sympathetic nerves, and an enhancement of the cerebrovascular response to CO_2 following cervical sympathectomy. Also, D'Alecy & Feigl (1972) reported significant cerebral vasoconstriction following stimulation of the stellate ganglion in the anaesthetized dog; this response was blocked by α-adrenoceptor antagonists (D'Alecy, 1973).

The results of experiments with vasoactive drugs are also conflicting. Carpi (1972) reviewed 42 papers in which the effect of adrenaline or noradrenaline (NA) on cerebral blood flow was examined. No consistent pattern emerges. If anything, more authors have reported a vasodilator effect of these two drugs than vasoconstriction. Most of these experiments have involved the intravenous or intracarotid infusion of catecholamines, often in anaesthetized animals. Some of the variability in the results may be due to the anaesthetics used, to extra-cranial effects particularly where there are rich anastomotic channels between the intra- and extra-cranial vessels, to autoregulatory responses to the systemic blood pressure effects of these drugs particularly when given intravenously, or possibly, to difficulty of access of these drugs to adrenoceptors which probably lie on the adventitial side of the media of the resistance vessels. Also, there is rapid and avid binding of NA by the nerves which supply cerebral blood vessels (Rosenblum, 1973), so that less may be available for stimulation of NA receptor sites on the vessels. Another possibility applicable to both sympathetic stimulation and catecholamine infusion, is that the effects may be non-uniform, i.e. involve a redistribution of blood flow from some areas to others. In this situation measurement of total cerebral blood flow is useless

In experiments designed to assess the effect of local injections of adrenoceptor agonists on hypothalamic blood flow (HBF) in conscious rabbits, it has been shown (Rosendorff, 1972) that small doses of NA cause vasodilatation and larger doses local vasoconstriction. These effects were abolished by the addition of β- or α-adrenoceptor antagonists respectively, suggesting that there are adrenoceptors of both types in cerebral resistance vessels However, the doses used were likely to produce local concentrations of NA considerably higher than those following the release of endogenous transmitter. We have, therefore, attempted to answer the question. Do adrenoceptors in or around resistance vessels within the brain respond to released endogenous transmitter?

Methods

The area of brain studied was the hypothalamus, chosen because it is a homogeneously perfused region rich in grey matter with a relatively high concentration of endogenous NA. The technique for measuring local blood flow in the hypothalamus of the conscious rabbit has been described in detail elsewhere (Cranston & Rosendorff, 1971; Rosendorff & Cranston, 1971; Rosendorff, 1972). The method is a modification of that described by Monnier & Gangloff (1961); headplates with holes drilled at 3 mm intervals were screwed to the skulls of New Zealand White rabbits anaesthetized with pentobarbitone and weighing 2.5-3.0 kg. Holes were also drilled through the skull at co-ordinates aA and aB (Monnier & Gangloff, 1961) on both sides.

At the time of the experiments, not less than a week later, injection cannulae were placed so that their tips lay in identical positions in the hypothalamus on either side of the midline. Local blood flow was determined by injection of 15 μCi of ^{133}xenon dissolved in 5 μl of 0.9% w/v NaCl solution (saline) into the hypothalamus. Following each injection, the clearance of the radioactive isotope was measured with an external, collimated scintillation detector, a ratemeter and pulse-height analyser, a logic and routing assembly, and recorded on an ultraviolet recorder, teletype printer and punched tape. The hypothalamic blood flow (HBF, as ml 100 g^{-1} min^{-1}) was then computed from the formula, $HBF = \lambda \,.\, \log_e 2/T\frac{1}{2}$, where $\lambda = 0.74$, the brain tissue to blood partition coefficient for the rabbit hypothalamus (Rosendorff & Luff, 1970), and $T\frac{1}{2}$ = the half-decay time of the clearance curve. HBF values were obtained from an IBM 360/50 computer programmed to produce a best-fit regression of log counts above background vs time (0 to 100 seconds). Analysis of HBF values was in all cases done from monoexponential clearance curves. Curves with straight line correlation coefficients of less than 0.85 were rejected. This is considerably more rigorous than the 0.77 required for significance at the 1% level of confidence This ensured that only steady-state flows, at least during the period of xenon clearance, were analysed. The number of flow rates measured was 548 of which only 31 (5.7%) were rejected because of a correlation coefficient less than 0.85.

One side of the hypothalamus was the control side (^{133}Xe in saline only) and the other side was the test side (^{133}Xe in saline plus the tests substance). Injections were made into each side alternately, at intervals of at least 10 minutes. Changes in blood flow on the test side were calculated by subtraction of the mean of the control flows preceding and succeeding the test flow, from the test flow. The change in flow was then expressed as a positive or negative value relative to the control flows. Three to eight injections were made into each side during any one experiment. Previously (Rosendorff, 1972) it has been shown that there was no systematic difference in HBF between the two sides of the midline if no vasoactive substances were injected. Also it has been demonstrated that, at least within the volume of distribution of the injected ^{133}Xe, hypothalamic resistance vessels autoregulate within the mean arterial blood pressure range of 40-140 mmHg and retain CO_2-sensitivity (Cranston & Rosendorff, 1971).

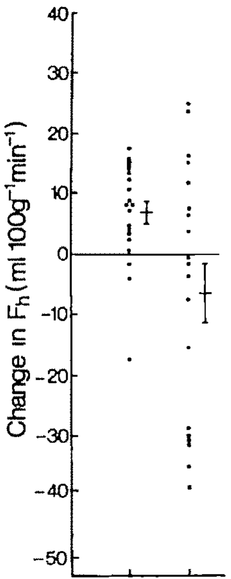
Drugs

The drugs tested were the β-adrenoceptor agonist isoprenaline (Isuprel, Winthrop), the β-adrenoceptor blocker propranolol (Inderal, I.C.I.), the α-adrenoceptor blocker phenoxybenzamine (Dibenyline, Smith, Kline & French) and tyramine (4-hydroxyphenethylamine, Sigma). Tyramine causes the release of endogenous NA from noradrenergic nerve terminals (Burn & Rand, 1958). In some experiments, detailed below, local chemical sympathectomy was performed by the use of 6-hydroxydopamine (Sigma), and in others, reserpine (Serpasil, Ciba) was used to deplete nerve terminals of biogenic amines (Rosenblum, 1973).

Results

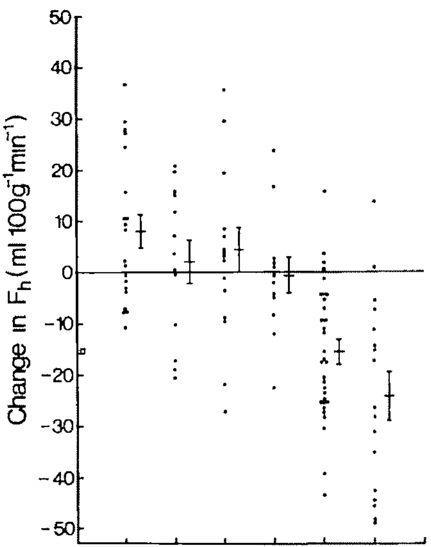
Isoprenaline

The presence of a β-receptor mediated vasodilatation was established by injection of isoprenaline



µg/inj	04	04 + Prop 20µg
n	20	19
x̄	69	-64
se mean	19	48
P	<0 005	NS

Figure 1 Effect of isoprenaline on hypothalamic blood flow (F_h) in the conscious rabbit. Abscissae: dose of isoprenaline, and isoprenaline plus 20 µg propranolol (Prop). Ordinates. F_h compared to mean control values (see text). Note that propranolol abolishes vasodilatation.
n = number of ^{133}Xe curves on the test side; $\bar{x}$ = mean F_h; P = significance level for the difference of each test mean from control mean (= 0) by Student's t test.



µg/inj	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	1
n	20	13	16	12	32	17
x̄	80	20	43	-07	-156	-243
se mean	32	41	44	35.	23	47
P	<0025	NS	NS	NS	<0001	<0001

Figure 2 Effect of tyramine on hypothalamic blood flow (F_h) in the conscious rabbit. Abscissae: dose of tyramine in µg per injection. Ordinates: F_h compared to control values (see text). Vertical bars represent the s.e. mean. Note that the 10^{-5} µg (10 pg) per injection dose of tyramine causes an increase in mean F_h, while the 10^{-1} (100 ng) and 1.0 µg per injection doses cause a decrease in mean F_h
n = number of ^{133}Xe curves on test side; $\bar{x}$ = mean F_h; P = significance level for the difference of each mean from control flows (= 0) by Student's t test.

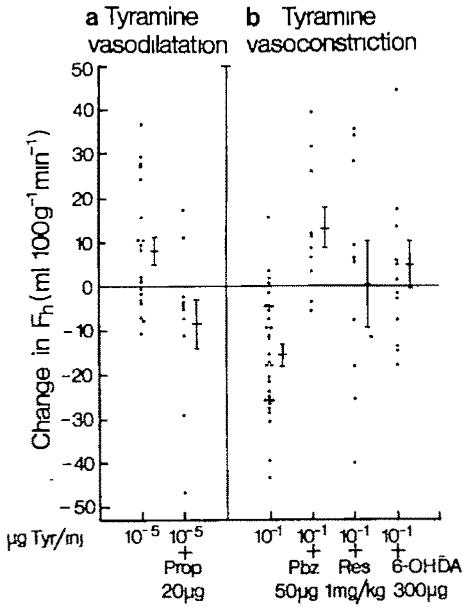
(0.4 µg/injection). Isoprenaline produced a significant ($P < 0.005$) mean increase of 6.9 ml 100 g tissue^{-1} min^{-1} in HBF (Figure 1). This effect was abolished by the addition of 20 µg of propranolol to the injectate (Figure 1).

Tyramine

Figure 2 shows the dose-response relationship for tyramine. The smallest dose tested (10 pg per injection) produced an increase in mean HBF of 8.0 ml 100 g^{-1} min^{-1}, while doses of 100 ng and 1.0 µg per injection reduced HBF by mean values of 15.6 and 24.3 ml 100 g^{-1} min^{-1}, respectively. Intermediate doses had no significant effect.

The increase in HBF produced by the 10 pg dose of tyramine could be abolished by the addition of 20 µg propranolol to the injectate. The flow was reduced to 8.5 ml 100 g^{-1} min^{-1} less than the control side, which was significantly different ($P < 0.02$) from the response produced by the 10 pg dose of tyramine alone (Figure 3). This result suggests that a β-adrenoceptor responds to endogenous noradrenaline released from nerve terminals by small doses of tyramine.

The vasoconstrictor effect of 100 ng tyramine per injection was abolished, and possibly reversed, by the addition of 50 µg of phenoxybenzamine to the injectate (Figure 3). After the addition of phenoxybenzamine to the injectate the HBF increased to a mean value of 13.2 ml 100 g^{-1} min^{-1} ($P < 0.025$) greater than control flows. Depletion of biogenic amine stores by daily intramuscular injection of reserpine 1 mg/kg for three days also abolished the vasoconstrictor effect of tyramine as did 6-hydroxydopamine (300 µg)



n	20	10	32	10	10	13
$\bar{x}$	80	-85	-156	131	01	50
se mean	32	56	23	47	100	51
P	<0025	<002	<0001	<0025	NS	NS

Figure 3 (a) Effect of propranolol (Prop 20 µg per injection) added to the 10^{-5} µg (10 pg) tyramine (Tyr) injection. The mean F_h is changed from +8.0 ± 3.2 ml 100 g^{-1} min^{-1} to −8.5 ± 5.6 ml 100 g^{-1} min^{-1}. These two results differ significantly ($P < 0.02$).

(b) Effect of phenoxybenzamine (Pbz 50 µg per injection) added to the 10^{-1} µg (100 ng) tyramine injection The mean F_h is changed from −15.6 ± 2.3 ml 100 g^{-1} min^{-1} (which is significantly less than control = 0, $P < 0.001$, $n = 32$) to +13.1 ± 4.7 ml 100 g^{-1} min^{-1} (which is greater than control = 0, $P < 0.025$, $n = 10$). Pretreatment with reserpine or 6-hydroxydopamine (6-OHDA) (see text), abolishes the vasoconstrictor effect of 10^{-1} µg of tyramine.

injected into the hypothalamus 4 days before the experiment. These results suggest that vasoconstriction is dependent on α-adrenoceptor activation by NA released from nerve terminals by the larger doses of tyramine.

Discussion

Pial and other arteries have a dense adrenergic innervation and respond to sympathetic stimulation and denervation. The major controversy relates to the intracerebral (intraparenchymal) resistance vessels Harper, Deshmukh, Rowan & Jennett (1972) have suggested that there is no autonomic control of the intracerebral vessels, and that these vessels are influenced only by metabolic factors. Sympathetic stimulation would result in constriction of extracerebral vessels only, and since extra- and intracerebral resistance vessels are in series, this will cause a fall in intracerebral arterial pressure. An autoregulatory adjustment would then cause the intracerebral vessels to dilate, and this tends to maintain an adequate flow. One piece of evidence in support of this idea is that the cerebral blood flow response to sympathetic stimulation would be more marked when the intracerebral vascular resistance was already reduced, e.g. by hypercapnia Several studies (Meyer, Gotoh, Akiyama & Yoshitake, 1967; James *et al.*, 1969; Harper *et al.*, 1972) have confirmed this.

However, we have demonstrated both previously and in this study, the existence of adrenoceptors associated with intracerebral vessels at least in the hypothalamus. The present results also suggest that these receptors respond to the release of endogenous NA. The mechanism of this response is still unresolved. It is possible that there are both α- and β-receptors on or close to the vascular smooth muscle. There is, however, an alternative explanation for these findings. Recent studies have shown that NA, applied to neurones by microiontophoresis, may increase (Boakes, Bradley, Brooks & Wolstencroft, 1968, Johnson, Roberts & Straughan, 1969) or decrease (Biscoe & Straughan, 1966; Phillis & York, 1967) the firing rate. Neuronal excitation has also been reported with isoprenaline (Johnson, Roberts, Sobieszek & Straughan, 1969), and NA excitation may be blocked by α- and β-adrenoceptor antagonists, and more consistently with β-antagonists (Brawley & Johnson, 1973).

The results described in this paper are therefore also compatible with the idea that one or both of the α- and β-adrenoceptors are extravascular, possibly on neuronal membranes, and that the action of the α- and β-stimulating agents may be primarily on neurones. The vasomotor effects might be secondary to a change in local metabolic activity induced by changes in neurone firing rates. This hypothesis is more likely to apply to the β-mediated vasodilatation; NA-induced inhibition of neurones sufficient to cause the large reduction in HBF of 15.6 ml 100 g^{-1} min^{-1} is probably less likely. This approach has, on the other hand, been criticized by Stone (1971) who suggests that the neuronal effects are secondary to the changes in vascular calibre. However, irrespective of the mechanism our results suggest that intracerebral blood flow, at least in the hypothalamus, is likely to be affected by released transmitter from noradrenergic nerve terminals.

This work was supported by the University of the Witwatersrand Council Research Committee, the Medical Research Council and the Atomic Energy Board of South Africa.

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(Received November 12, 1974)

Br. J. Pharmac. (1975), 54, 17-21

EFFECT OF CLONIDINE ON THE EXCITABILITY OF VASOMOTOR LOCI IN THE CAT

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1 The effect of clonidine on the direct excitability of hypothalamic, medullary and spinal vasomotor loci has been investigated in cats anaesthetized with chloralose.

2 Clonidine inhibited the excitability of these loci when it was localized to the central sites by intracerebroventricular, intravertebral arterial or intrathecal injection in very low doses (1-2 μg).

3 Topical application of clonidine (0.01% and 1.0%) to the floor of the fourth ventricle inhibited pressor responses evoked either by stimulation of medullary or hypothalamic vasomotor areas. Inhibition of the pressor responses was accompanied by hypotension and bradycardia in many experiments.

4 It appears that effects of clonidine on the vasomotor loci of the medulla oblongata and the spinal cord contribute to its hypotensive action.

Introduction

Intravenous administration of clonidine (Catapres) produces initial sympathomimetic effects by stimulating α-adrenoceptors (Maling, Cho, Horakova & Williams, 1969) followed by hypotension and bradycardia which have been ascribed to a central action (Sherman, Greca, Woods & Buckley, 1968; Shaw, Hunyor & Korner, 1971). Hukuhara, Otsuka, Tadeka & Sakai (1968) and Schmitt & Schmitt (1969) reported an inhibition of the action potentials in the splanchnic and cardiac nerves following central administration of clonidine. They localized the site of this action of clonidine to the medulla oblongata. Recently Boudier & Van Rossum (1972) reported that the injection of clonidine into the rat hypothalamus produced hypotension. The present study was undertaken to obtain direct evidence of the effect of clonidine on the excitability of the hypothalamic, medullary and spinal vasomotor loci.

Methods

All experiments were performed in adult cats (2.5-4.0 kg) of either sex. They were anaesthetized with chloralose (80.0 mg/kg, i.v.), vagotomized and put on artificial positive pressure respiration as a routine. One of the femoral veins was cannulated with a polythene tube. Blood pressure was recorded from the left femoral artery either through a Statham P23dc pressure transducer on a Grass Model 7 polygraph or through a mercury manometer on a kymograph. In some experiments heart rate was also recorded through a Grass 7P4 tachograph preamplifier.

Effect on hypothalamic and medullary vasomotor loci

Hypothalamic and medullary vasomotor loci were stimulated electrically (0.5-4 V, 1 ms, 100 Hz for 5 s) by means of stereotaxically placed bipolar concentric stainless steel needle electrodes insulated except for 0.5 mm at their tips. Placement of the electrodes was aided by the coordinates given by Jasper & Ajmone-Marson (1954) as follows: for hypothalamus, frontal 9-10 mm, lateral 1-2 mm and depth 4-6 mm; for medulla, caudal 8-10 mm, lateral 2-4 mm and depth 4-6 mm. The depth was fixed by repeated stimulation after slowly driving the electrode into the tissue and choosing the most reactive point. Square wave pulses were derived from an Aplab electronic stimulator. A series of voltages were used to obtain graded vasomotor responses. The smallest voltage giving a clearcut response was called a threshold response while a larger submaximal response was called an optimal response. The drug was not given until these control responses were constant.

Clonidine was administered into the left lateral ventricle, through a Collison cannula implanted

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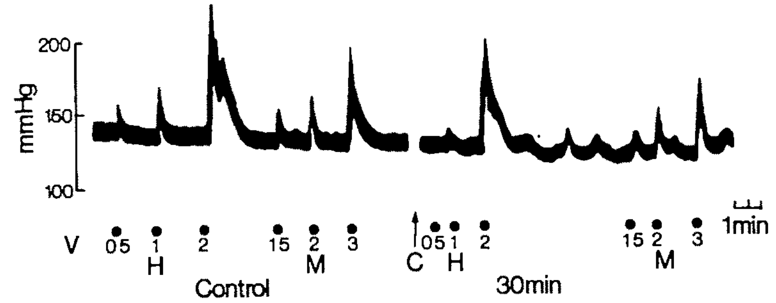

Figure 1 Blood pressure of a 3 kg cat anaesthetized with chloralose. Pressor responses to 0.5 (14 mmHg), 1.0 (34 mmHg) and 2.0 V (88 mmHg) stimulation of hypothalamic (H) and 1.5 (20 mmHg), 2.0 (32 mmHg) and 3.0 V (66 mmHg) stimulation of medullary (M) vasomotor areas. Clonidine (1 μg i.c.v.) was administered at C. Note the inhibition of hypothalamic responses to 0, 11 and 75 mmHg respectively and the medullary vasomotor responses to 14, 30 and 52 mmHg respectively at 30 minutes. There is no significant change in the blood pressure level.

according to the technique of Feldberg & Sherwood (1953), in a volume of 0.15 ml followed by 0.10 ml of 0.9% w/v NaCl solution (saline).

In some cats the drug was administered into a vertebral artery by means of a polythene tube introduced through the brachial artery with its tip close to the origin of the verebral artery. All branches of the subclavian artery except the common carotid were ligated. At the time of injection of the drug the common carotid artery was temporarily occluded.

The effect of the topical application of the drug to the floor of the IVth ventricle on the medullary and hypothalamic responses was studied in other cats. Part of the occipital bone was removed and the dura was cut away. The cerebellum was then pushed upwards to expose the floor of the fourth ventricle. Medullary and hypothalamic loci were electrically stimulated as described above and a cotton pledget soaked in the drug solution was applied at the site of the medullary electrode.

Effect on spinal vasomotor loci

The effect of the drug on the excitability of the spinal vasomotor neurones was assessed by eliciting spinal compression vasomotor responses (SCVR). The spinal cord was ligated within the meninges at C-7. A hypodermic needle, attached to a pressure bottle filled with saline for transmitting fluid compression to spinal cord and connected in parallel with an aneroid manometer, was introduced into the theca at the lumbosacral articulation. Spinal compression (100-150 mmHg) for 10-20 s elicited a consistent pressor response (White & Bonson, 1955). The drug was injected intrathecally in a volume of 0.2 ml followed by 0.1 ml of saline through the same needle which was used for spinal compression.

Whenever possible, mean alteration from control responses has been calculated and the standard error of the mean (s.e.) determined. The values shown in the results are mean values ± s.e.

Results

Effect of intracerebroventricular administration

Intracerebroventricular injection of 1-2 μg clonidine in 10 cats inhibited the hypothalamic as well as medullary vasomotor responses for 90-120 minutes. With an intracerebroventricular dose of 1 μg the hypothalamic threshold vasomotor response was inhibited by 41 ± 16.3% whereas an optimal vasomotor response was inhibited by 44 ± 12.3%. The same dose of clonidine inhibited the medullary threshold response by 50 ± 12.3% and the optimal response by 36 ± 8.3%. The inhibitory effect started in 5-10 min and reached a peak in 20-40 minutes. Inhibition of the vasomotor responses was accompanied by a mild hypotension (8.0 ± 7.9 mmHg) (Figure 1).

Effect of intravertebral arterial injection

Intravertebral arterial injection of clonidine in 6 cats (1-2 μg) produced essentially the same effect as produced by intracerebroventricular administration. Drug administration was followed within 5-10 min by inhibition of hypothalamic as well as medullary pressor responses and bradycardia. The effect of the compound lasted 20-60 minutes. In

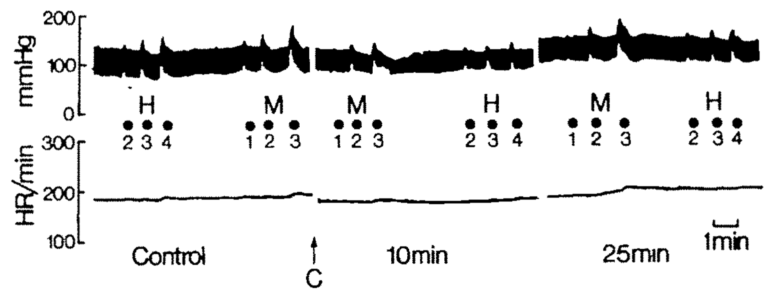

Figure 2 Blood pressure (mmHg) and heart rate (HR/min) of a 2.8 kg cat anaesthetized with chloralose. Hypothalamic (H) and medullary (M) vasomotor areas were stimulated by the voltages indicated at various points in the figure. Clonidine (2 μg) was injected into a vertebral artery at C. Blood pressure was lowered by 15 mmHg, bradycardia (20/min) occurred and responses were inhibited to hypothalamic stimulation (from 12, 20 and 35 to 8, 12 and 16 mmHg) and medullary stimulation (from 10, 30 and 45 to 8, 12 and 16 mmHg) at 10 min (middle panels) Partial recovery occurred after 25 min (last panels)

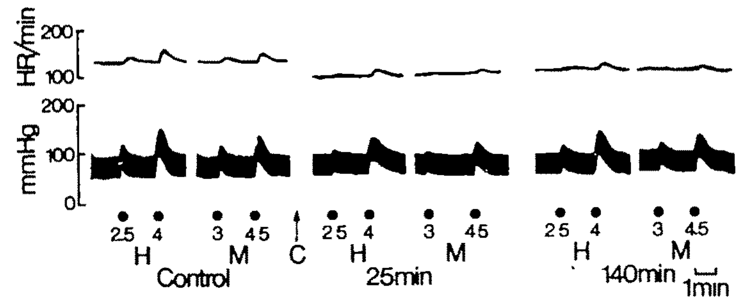

Figure 3 Heart rate (HR/min) and blood pressure (mmHg) of a 3 kg cat anaesthetized with chloralose. Threshold and optimal vasomotor responses to stimulation of hypothalamic (H) and medullary (M) vasomotor areas at the voltages indicated in the figure are shown. Clonidine 0.01% (C) was applied topically to the floor of the IVth ventricle. Note bradycardia (30/min) and inhibition of all the responses (hypothalamic pressor responses decreased from 30 and 55 to 10 and 35 mmHg and medullary pressure responses decreased from 25 and 40 to 5 and 25 mmHg respectively) at 25 min (middle panel). There was partial recovery after 140 minutes.

some experiments a weak hypotensive effect was also observed (Figure 2).

Effect of topical application

Topical application of clonidine (0.01% solution) to the floor of the fourth ventricle was followed by inhibition of threshold as well as optimal medullary and hypothalamic pressor responses within 10-20 min in 15 cats. Bradycardia also occurred. The medullary threshold pressor response was inhibited by 67 ± 16.2% whereas optimal pressor response was inhibited by 50 ± 9.2%. An inhibition (71 ± 6.5% and 62 ± 9% respectively) of hypothalamic pressor responses was also observed. Inhibition of responses was not accompanied by any significant hypotension in most of the cats (Figure 3). Recovery of the vasomotor responses occurred in 140 to 200 min after the removal of the drug-soaked cotton pledget. Topical application of a concentrated (1.0%) solution of clonidine produced hypotension (23 ± 11.9 mmHg) in addition to the inhibition of the medullary threshold (69 ± 12.9%) and optimal (53 ± 13.3%) vasomotor responses. The hypothalamic threshold and optimal vasomotor responses were inhibited by 95 ± 8.2% and 88 ± 11% respectively. With the higher concentration of clonidine the inhibition of the responses was obtained earlier (5-10 min) and persisted much longer (more than 180 minutes).

Effect on spinal vasomotor neurones

Intrathecal injection of 1-2 μg clonidine inhibited threshold as well as optimal SCVR in 7 cats. The threshold and optimal responses were inhibited by 44.5 ± 7.2% and 46 ± 8.6% respectively. Peak

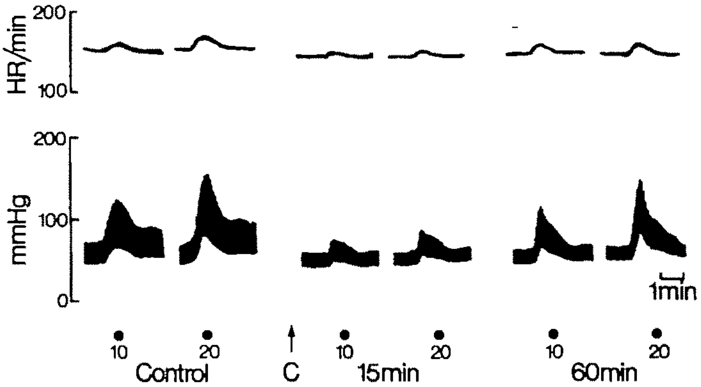

Figure 4 Heart rate (HR/min) and blood pressure (mmHg) of a 3.2 kg spinal transected cat. Spinal compression vasomotor responses (SCVR) were elicited by a 10 and 20 s compression. Clonidine, 1 μg (C) inhibited SCVR at 15 min from 65 and 85 to 25 and 35 mmHg respectively (mddle panel) Recovery occurred at 60 min (last panel).

effect was evident in 5-15 min and recovery occurred in 60-120 minutes. There was no significant change in the blood pressure (Figure 4).

Discussion

Several authors have suggested a central mechanism in the hypotensive action of clonidine (Sherman *et al.*, 1968; Schmitt & Schmitt, 1969; Shaw *et al.*, 1971). However, their studies do not provide adequate information about the direct effects of clonidine on the excitability of neurones in central cardiovascular loci. In the present investigation the effect of clonidine on the excitability of these neurones, as judged by responses to electrical stimulation (hypothalamus, medulla) or pressure (spinal cord), has been studied because these stimuli give reproducible blood pressure and heart rate changes for several hours (Gupta, Srimal & Dhawan, 1972).

The present findings demonstrate an inhibitory effect of clonidine on the excitability of central vasomotor loci. The inhibitory action seems to be mainly exerted at the medullary and the spinal levels. An action at the medullary level has also been proposed by Schmitt & Schmitt (1969). The spinal cord is being increasingly recognized as a possible central site for cardiovascular effects (Smith, 1974) and it is interesting to note that the spinal loci are also inhibited by clonidine.

Bousquet & Guertzenstein (1973) reported a rise of blood pressure in the cat due to the action of clonidine on the hypothalamus and localized the hypotensive action of this drug to the medulla oblongata. The dose employed by these authors was quite high (10-100 μg i.c.v.) compared to the doses (1-2 μg) used in our experiments. Our results agree with these authors as far as the action of clonidine on the medulla is concerned. We have made no attempt to localize the compound to the hypothalamus alone. It is likely that the inhibition of hypothalamic responses following intracerebroventricular or intravertebral arterial administration of clonidine in our experiments is due to an action at the medullary level since similar inhibition is also observed following topical application of the drug to the floor of the IVth ventricle (Figure 3). However, Boudier & Van Rossum (1972) reported hypotension and bradycardia following local injection of clonidine into the hypothalamus of the rat. This variation in result may be due to a species difference.

An analysis of the results obtained in the present study clearly indicates that the inhibition of excitability of vasomotor neurones can be obtained even with doses of clonidine which do not produce hypotension in our experiments. Similar findings have been reported by us with 6-hydroxydopamine (Gupta *et al.*, 1972) and α-methylnoradrenaline (Singh, Srimal & Dhawan, 1973). We have also found (Bapat, Dhawan & Srimal, 1969) that (−)-N-(1-phenylethyl)-guanidine could facilitate centrally evoked pressor responses in a dose which had no effect on the blood pressure. Higher doses produced a rise in blood pressure as well. It appears that alteration in the excitability of vasomotor neurones is a more sensitive index of drug action on the central cardiovascular loci than changes in the blood pressure level.

Starke & Montel (1973) reported a decrease in the output of noradrenaline due to electrical stimulation of cerebral neurones following clonidine administration. They attributed this decrease in output to stimulation of the α-adrenoceptors by clonidine. It is likely that there is an adrenergic link in the pathway which mediates the pressor responses obtained due to stimulation of the vasomotor loci and the decrease in the output of the transmitter is due either to stimulation of the α-receptors (clonidine) or to blockade of the adrenergic transmission (6-hydroxydopamine; Gupta *et al.*, 1972) which results in the decrease of the vasomotor responses and may ultimately, in higher doses, produce hypotension.

There has been a divergence of opinion regarding the role of central effects of clonidine in the hypotension observed following therapeutic doses of this drug. Because Katic, Lavery & Lowe (1972) found neither hypotension nor any evidence of central inhibitory action of clonidine (20 μg/kg) in dogs on chronic oral administration, they suggested that the central effect of clonidine was unimportant for producing hypotension in therapeutic doses. However, since the dose was without any effect, it can hardly be used as evidence against a central action. On the other hand Sherman *et al.* (1968) consider the central action of clonidine to be quite significant in producing hypotension. In the present series of experiments, effects have been obtained following central administration of low doses of clonidine which have no effect when injected peripherally. It is reasonable to assume that such small amounts could reach the central nervous system following administration of clonidine in therapeutic doses. The inhibition of central vasomotor loci is, therefore, likely to contribute significantly to the hypotension and bradycardia following systemic administration of clonidine.

This is Communication No. 1848 from the Central Drug Research Institute, Lucknow, India. D.V was supported by the University Grants Commission, New Delhi and M.B.J. by the Indian Council of Medical Research, New Delhi, India. Clonidine was kindly donated by Boehringer, Ingelheim, Germany.

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(Received September 23, 1974.
Revised January 6, 1975)

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Br. J. Pharmac. (1974), 54, 23-31

ACETYLCHOLINE-SENSITIVE CELLS IN THE CAUDAL MEDULLA OF THE RAT: DISTRIBUTION, PHARMACOLOGY AND EFFECTS OF PENTOBARBITONE

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1 The distribution of cholinoceptive and non-cholinoceptive cells in various nuclei of the caudal medulla of the rat is described.

2 The nature of the responses of cells of the paramedian reticular nucleus and of the perihypoglossal nuclei to electrophoretically applied acetylcholine (ACh) was investigated.

3 In unanaesthetized decerebrate preparations ACh responses were usually of a 'fast onset-fast offset' nature. Dihydro-β-erythroidine was a more effective antagonist than atropine.

4 In rats anaesthetized with barbiturate nearly all the ACh responses showed a slower onset and prolonged action. Atropine was the more effective antagonist.

5 The synaptic responses of cells of the paramedian reticular and perihypoglossal nuclei to stimulation of glossopharyngeal, superior laryngeal, lingual and hypoglossal nerves were investigated. It is concluded that ACh does not mediate the responses at the level of these nuclei.

Introduction

Previous reports concerning acetylcholine (ACh)-sensitive cells in the medulla of the cat (Salmoiraghi & Steiner, 1963; Bradley, Dhawan & Wolstencroft, 1966; Avanzino, Bradley & Wolstencroft, 1966; Yamamoto, 1967) and of the rat (Bradley & Dray, 1972, 1973) contain little data on the synaptic connections of these cells or on their location in different nuclear groups. However, in the cat Avanzino *et al.* (1966) found that ACh excited almost all those cells in the region of the paramedian reticular nucleus which were antidromically activated from the cerebellum.

Excitation of medullary reticular cells by ACh has been reported in the rat anaesthetized with barbiturate and in unanaesthetized animals (Bradley & Dray, 1973) and it was suggested that the short latency excitation observed in decerebrate rats may subserve a synaptic function.

In the present experiments an attempt was made to relate excitation of identified rat medullary cells by ACh to a synaptic input. In addition, the nature of the response to ACh and the specificity of nicotinic and muscarinic antagonists in rats anaesthetized with barbiturate were found to be different from those in decerebrate preparations.

Methods

Rats were either anaesthetized with pentobarbitone sodium 45 mg/kg intraperitoneally, supplemented with intravenous injections of a 10 mg/ml solution, or were decerebrated under halothane anaesthesia. The latter involved electrolytic coagulation of the midbrain followed by suction of cerebral tissue rostral to the coagulated area. Both common carotid arteries were tied to minimize blood loss during this procedure. Anaesthesia was discontinued after decerebration. All animals were artificially ventilated with humidified oxygen following muscle relaxation with gallamine triethiodide.

The brain stem was approached ventrally as previously described (Duggan, Lodge & Biscoe, 1973). The following nerves were prepared for stimulation as a means of identifying cell groups in the medulla: superior laryngeal, recurrent laryngeal, glossopharyngeal, hypoglossal and lingual nerves.

Compounds were administered electrophoretically from the outer barrels of seven barrel micropipettes. These compounds, together with their concentrations and pH, were: L-glutamate Na (Koch-Light), 0.5 M pH 8; DL-homocysteate Na (Koch-Light), 0.2 M pH 8; acetylcholine bromide (BDH), 0.5 or 1.0 M pH 3.3; acetyl-β-methylcholine bromide (Koch-Light), 0.5 M pH 3.7; atropine sulphate (Koch-Light), 10 mM in 165 mM NaCl pH 4.6; dihydro-β-erythroidine (Merck, Sharpe & Dohme), 0.1 M pH 4.6; pontamine sky blue (G.T. Gurr), 2% in 0.5 M Na acetate pH 7.7. A retaining voltage of 0.5 V was routinely applied to each barrel except that containing the dye (see Lodge, Caddy, Headley & Biscoe, 1974); however, lower retaining voltages were frequently used on barrels containing the excitants so as to minimize the latency to the onset of their actions from the beginning of the application of an ejecting voltage.

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Action potentials were recorded with the 4 M NaCl filled centre barrels of the micropipettes, and by conventional techniques the firing rates of single cells were displayed continuously on a potentiometric pen recorder. The synaptic activation of cells was studied quantitatively by preparing poststimulus histograms using a small computer (Biomac 500).

During the course of each experiment, physiological landmarks, e.g. the field potential from antidromic activation of hypoglossal motoneurones, as well as the usual micromanipulator readings, were used as guides to the position of the microelectrode tip. Pontamine sky blue was ejected at stereotaxically defined recording sites, and the resultant blue spots were found by cutting 25 or 50 μm frozen sections of the formalin-fixed brain stem. Thus by a combination of micromanipulator readings and histologically identified dye marks, the locations of most of the cells investigated were plotted on outline drawings of serial brain stem sections.

Results

The present study describes the location of ACh-sensitive cells in various nuclei in the caudal medulla of the rat. An attempt was made to correlate sensitivity to ACh with synaptic inputs; also the effect of ACh antagonists on synaptic transmission was tested on cholinoceptive cells. During the course of this investigation a previously undescribed difference in the pharmacology of cholinoceptive cells in the paramedian reticular and perihypoglossal nuclei was found between decerebrate and barbiturate-anaesthetized rats.

(1) Distribution of cholinoceptive cells in the medial part of the caudal medulla

Cells were located by their spontaneous discharge, by their response to the ejection of ACh or an excitant amino acid, or by their response to stimulation of the prepared cranial nerves. The distribution of 109 such neurones in tracks not more than 0.9 mm lateral to the midline is shown in Figure 1. No attempt was made to estimate the proportions of cholinoceptive and non-cholinoceptive cells in any particular area as the problems of sample bias make such estimates inaccurate. However, certain generalizations about the responses to ACh of various cell groups in this region can be made.

(a) Paramedian reticular and perihypoglossal nuclei A large proportion of neurones in these areas project to the cerebellum (Brodal & Torvik, 1954). In three experiments such cells were identified by juxtafastigial cerebellar stimulation which caused both antidromic and synaptic excitation; examples of such responses are shown in Figures 4a & b. The short latency (1.1 ms) and the high following frequency (500 Hz) suggest that the spikes in Figure 4a and the first in Figure 4b were antidromically evoked whereas the second spike in Figure 4b probably resulted from orthodromic activation. In all other experiments the paramedian reticular nucleus and the perihypoglossal nuclei were identified only histologically. Many cells in this area were cholinoceptive; it seemed that such cells were sampled more readily in decerebrate rats, for of the 99 cholinoceptive neurones studied 70 were found in 10 decerebrate, and 29 in 11 barbiturate anaesthetized preparations.

(b) Hypoglossal nucleus Throughout this series of experiments many tracks of the electrode were made through the hypoglossal nucleus, the antidromic field of which served as a useful reference to electrode trip position (see methods section). No cholinoceptive cells were ever found.

(c) Inferior olivary nucleus The actions of ACh in this nucleus have been described in a separate communication (Duggan, Lodge, Headley & Biscoe, 1973). Neurones responded to ACh with a marginal increase in firing rate.

(d) Nucleus of the solitary tract Neurones in this nucleus were defined as those activated from a single stimulus to the glossopharyngeal or superior laryngeal nerve with a latency of between 2 and 5 ms. Such responses were usually superimposed on a large negative field potential (Fig. 4d) which helped to define the area. None of the five cells tested was cholinoceptive.

(e) More laterally located neurones Neurones of the nucleus ambiguus, antidromically evoked from

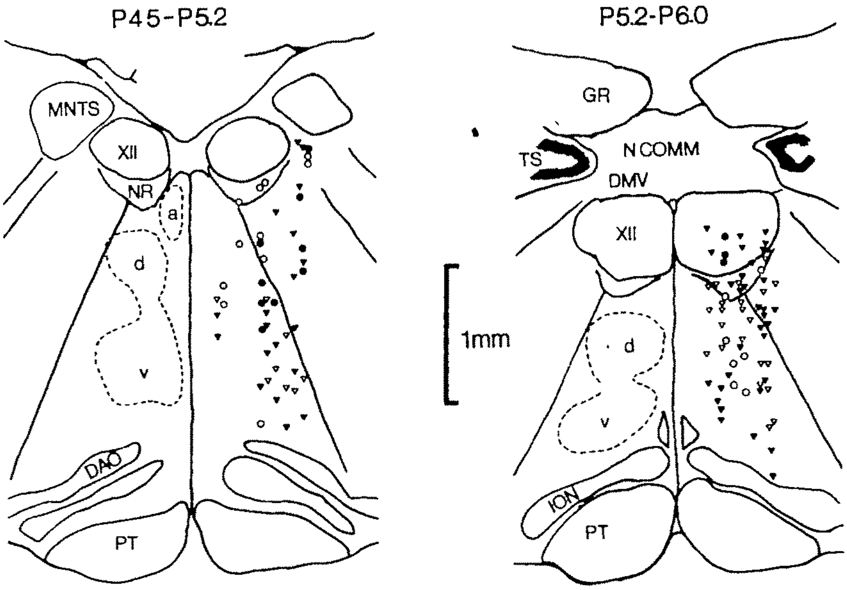

Figure 1 Distribution of cholinoceptive and non-cholinoceptive cells in the region of the paramedian reticular and perihypoglossal nuclei of the rat. The positions of cholinoceptive (open symbols) and non-cholinoceptive (closed symbols) neurones in both barbiturate anaesthetized (circles) and decerebrate (triangles) preparations, were projected onto the appropriate line drawing by reference to stereotaxic coordinates, pontamine sky blue marks and the antidromically evoked hypoglossal field depth profile. The figures above the section give the inclusive antero-posterior coordinates of each. a,d,v = accessory, dorsal and ventral parts of the paramedian reticular nucleus as proposed for the cat by Brodal & Torvik (1954); DAO = dorsal accessory olive; DMV = dorsal motor nucleus of the vagus; GR = gracile nucleus, ION = inferior olivary nucleus, N COMM = commissural nucleus of Cajal; NR = nucleus of Roller; PT = pyramidal tract; TS = solitary tract; XII = hypoglossal nucleus; MNTS = medial nucleus of the solitary tract.

the recurrent laryngeal nerve, were not cholinoceptive. Some physiologically unidentified neurones in the surrounding reticular formation were found to be excited by ACh.

No cells were studied in the sensory nucleus of the trigeminal nerve, the dorsal column nuclei, the motor nucleus of the vagus or in the commissural nucleus of Cajal.

(2) The nature of the response of neurones in the paramedian reticular and perihypoglossal nuclei to electrophoretically applied acetylcholine; comparison of decerebrate and barbiturate-anaesthetized preparations

The actions of ACh on central neurones of the cat fall broadly into two groups; a 'fast onset-fast offset' action typified by the nicotinic response of spinal Renshaw cells (Curtis & Ryall, 1966), and a slow onset-prolonged action as seen at the muscarinic receptors of cerebral cortical neurones (Krnjević & Phillis, 1963; Crawford & Curtis, 1966). In the present study a 'fast action' is defined as one in which peak firing frequency was reached within 10 seconds.

As the decerebrate and the barbiturate-anaesthetized animals differed in the types of response they will be described separately.

(a) Decerebrate preparations In the 10 decerebrate rats, 35 of 70 cholinoceptive cells showed that 'fast' type of response to ACh, whereas with 19 the 'slow' response was observed. On the remaining 16 neurones ACh had an excitant effect which had a variable latency and so was difficult to classify into these two groups. No depressant action either on spontaneous firing or on the firing in response to the ejection of L-glutamate was observed apart from that considered to be due to cationic current.

The nature of the ACh response of 17 neurones was further examined by ejecting dihydro-β-erythroidine (DHβE; 6-30 nA) and/or atropine (8-52 nA) during the alternate ejection of acetylcholine and L-glutamate or DL-homocysteate (DLH). Of the seven cells on which the actions of both DHβE and atropine were tested, four showed selective ACh antagonism by DHβE but not by atropine, three showed antagonism by both substances whereas none

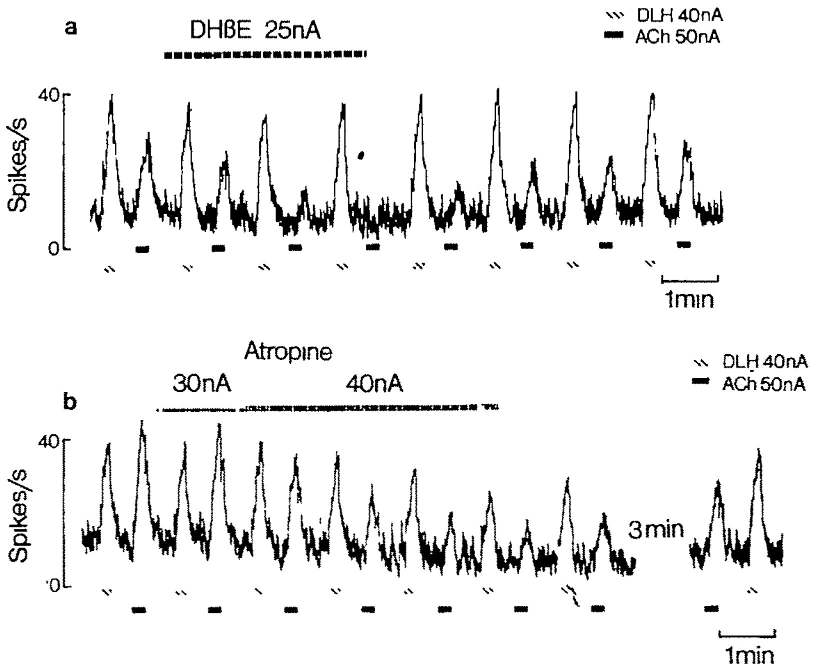

Figure 2 Effects of dihydro-β-erythroidine (DHβE) and atropine on acetylcholine (ACh) and DL-homocysteate (DLH) excitant actions on a perihypoglossal neurone in a decerebrate rat. The ratemeter records of (a) and (b) show consecutive tests on this cell which responded quickly to the ejection of ACh (50 nA for 15 seconds). The time constant of the ratemeter was 1 second. During the recovery from atropine 3 min of the trace have been omitted.

showed antagonism by atropine alone. On a further nine cells, DHβE alone was tested; reversible reduction of the ACh response alone was seen in five cells, and with three the action of ACh was reduced more than that of L-glutamate but full recovery was not observed. On the remaining cell, the only one of the 'slow' type, the ACh response was unaffected by DHβE (20 nA).

On one other cell atropine alone (up to 52 nA) was tested and no effect was observed until the responses to both excitants were reduced. Such high currents of atropine often resulted in reduction in spike amplitude (Curtis & Phillis, 1960).

The records of Figure 2 were obtained from a cell in the perihypoglossal nuclei. The action of acetylcholine was 'fast', being comparable in onset and decline to that of DLH. On this cell DHβE (25 nA) reversibly abolished the action of ACh without affecting that of DLH (Figure 2a), whereas atropine had no effect on the response to ACh until the actions of both excitants were reduced (Figure 2b).

Thus most cells in decerebrate preparations, had predominantly 'fast' ACh responses which on the basis of their sensitivity to the ACh antagonists, appeared to be mediated mainly by receptors of the nicotinic type.

(b) Rats anaesthetized with barbiturate In 11 such preparations ACh had a 'slow' action on 25 cells and a clearly 'fast' action on only 2 out of 29 cholinoceptive neurones studied. Not only was the time course of the responses longer than those seen in decerebrate preparations but ACh was a less potent excitant, relative to glutamate. In decerebrate preparations, the currents of ACh (20-60 nA, mean 44 nA) were usually similar to those of nearly equieffective currents of L-glutamate (16-70 nA, mean 39 nA), whereas in rats anaesthetized with barbiturate ACh ejecting currents (40-100 nA, mean 66 nA) needed to be two to three times as high as those of L-glutamate (8-56 nA, mean 26 nA) in order to produce excitant responses of a similar magnitude.

In rats anaesthetized with barbiturate, atropine (1-60 nA) was more effective as an ACh antagonist than was DHβE (8-180 nA). With all five cells tested with both antagonists, atropine selectively reduced the action of ACh whereas DHβE only did so on one. Atropine alone was tested on a further five cells and on only one was ACh antagonism not

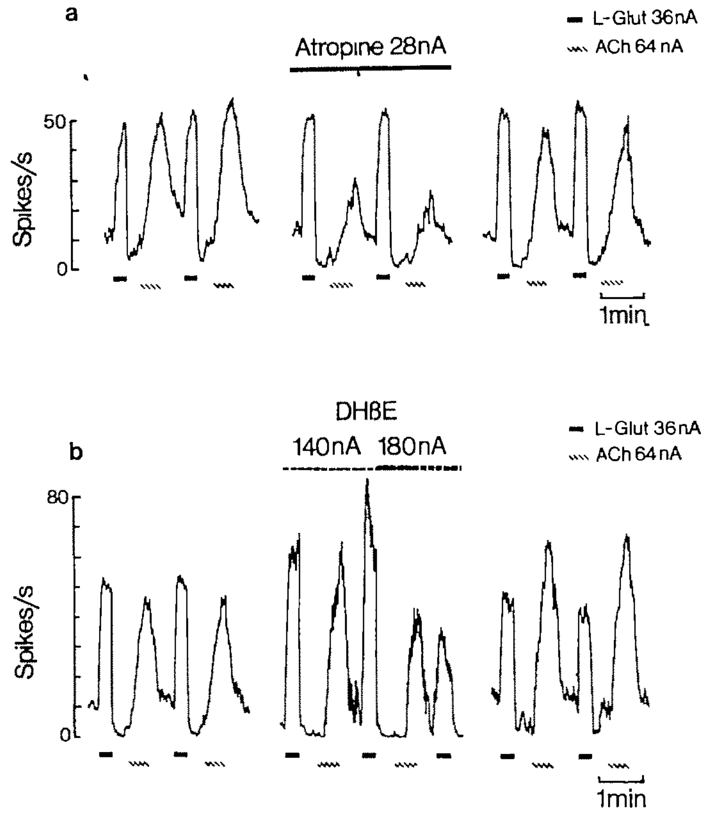

Figure 3 Effects of atropine and dihydro-β-erythroidine (DHβE) on acetylcholine (ACh) and L-glutamate (L-Glut) responses of a paramedian reticular neurone in a rat anaesthetized with barbiturate. This neurone responded only slowly to ACh requiring 30 s at 64 nA to reach a near-plateau firing rate. The action of L-glutamate (64 nA 15 s) was more rapid. Ratemeter time constant 0.3 s. (a) Is divided into three parts. The first shows control responses after which atropine was ejected with currents of 8 nA for 3 min and 16 nA for 1 5 minutes. The second part shows responses recorded whilst the atropine current was raised to 28 nA. The third part was taken 10 min after cessation of the atropine current. In (b), after the control response, the DHβE current was increased stepwise from 10 nA to 20, 30, 40, 60, 80 and 140 nA over a period of 15 min (not shown). The second part of the record shows the effect of the last part of 140 nA DHβE and the response when the current was raised to 180 nA. Recovery is shown 17 min after cessation of the DHβE current.

observed. On three other cells tested with DHβE alone, there was either no effect (two cells) or both ACh and glutamate responses were reduced (one cell).

Results from one rat anaesthetized with barbiturate are illustrated in Figure 3. Pontamine sky blue marking showed this cell to be located in the dorsal part of the paramedian reticular nucleus. The excitant action of ACh was slow in onset and prolonged by comparison with that of L-glutamate. An atropine current of 28 nA (Figure 3a) preferentially reduced the action of ACh; recovery is illustrated. On this same cell DHβE was ejected with increasing currents up to 180 nA; initially the responses to both excitants were increased (see 140 nA trace, Figure 3b), and then both were reduced in parallel. This effect has been seen on a number of occasions in the brain stem (see also section 4 below). The frequent occurrence of similar increases in the responses of rat Renshaw cells to excitants during the ejection of DHβE, has led us to conclude that this effect is related to the known direct excitant action of this drug (e.g. Curtis & Ryall, 1966).

The responses of cells in preparations anaesthetized with barbiturate thus show a marked difference from those in the decerebrate animals both in the nature of the response to ACh and in the susceptibility of this response to atropine and DHβE. The results suggest a preponderance of ACh receptors of the muscarinic type in the anaesthetized rats.

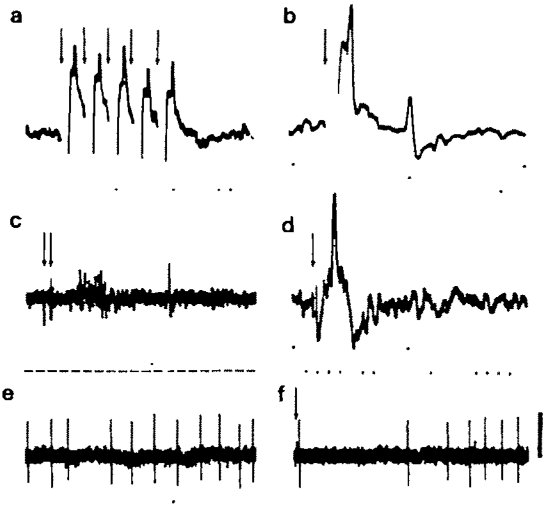

Figure 4 Activation of neurones in the area of the paramedian reticular nucleus. (a) Responses of a cholinoceptive cell just ventral to the hypoglossal nucleus to tetanic stimulation (500 Hz, 100 μA) near the contralateral fastigial nucleus. (b) Same cell as (a) showing response to a single shock (100 μA) near the ipsilateral fastigial nucleus. (c) Response of a cholinoceptive neurone in the ventral part of the paramedian reticular area to 2 stimuli (300 Hz, 100 μA) to the glossopharyngeal nerve. (d) Response of a non-cholinoceptive neurone in the region of the nucleus of the solitary tract to a single stimulus (1.5 V) to the glossopharyngeal nerve. (e and f) Slow sweep traces showing the steady firing pattern of a cell in the dorsal part of the paramedian reticular nucleus (e) during the ejection of acetylcholine 40 nA. In (f), the same firing rate is interrupted by a single stimulus (80 μA) to the ipsilateral superior laryngeal nerve. The calibration bar to the bottom right represents 200 μV for (a), (b) and (c) and 100 μV for (d), (e) and (f). The sweep durations illustrated are (a) 20 ms, (b) 10 ms, (c) 100 ms, (d) 20 ms, (e and f) 500 ms.

Acetyl-β-methylcholine was tested cursorily on seven cholinoceptive cells, and in each case had an effect similar to that of ACh, whether the responses to ACh were fast (two cells, decerebrate), intermediate (two cells, decerebrate) or slow (three cells, barbiturate).

(3) The synaptic responses of cells in the region of the paramedian reticular and perihypoglossal nuclei

(a) Glossopharyngeal nerve A single stimulus to the glossopharyngeal nerve seldom affected the firing rate of cholinoceptive cells. However a brief tetanus (2-3 stimuli of 50-150 μA and 0.15 ms duration, at 300 Hz) influenced the firing of 30 of the 39 cholinoceptive cells tested in decerebrate animals. Twenty cells were excited and 10 were inhibited.

The excitation usually consisted of a short train of spikes (Figure 4c). The latency of activation varied for any one cell but when poststimulus histograms were prepared the time of greatest probability of firing was normally seen to be between 15 and 30 milliseconds. This latency is greater than that of cells of the nucleus of the tractus solitarius evoked by a single shock to the glossopharyngeal nerve (Figure 4d). Whilst most cells of the paramedian region responded with a single burst of firing some followed this with a second, more prolonged burst.

The inhibitions were usually prolonged, often lasting several hundred milliseconds. An example is illustrated in Figure 4e & f; in this instance a single stimulus was sufficient to cause a prolonged inhibition. No intracellular or pharmacological studies to determine the nature of such inhibition have been attempted.

In animals anaesthetized with barbiturate, only two of eight cholinoceptive cells tested were activated by a brief tetanus to the glossopharyngeal nerve; one was inhibited.

In both types of preparation, stimuli to the glossopharyngeal nerve could also affect non-cholinoceptive cells of this region. The latency and the character of the evoked responses were similar to those of the cholinoceptive cells.

(b) Superior laryngeal nerve In decerebrate preparations, with stimulation parameters similar to those used with the glossopharyngeal nerve, 20 out of 25 cholinoceptive cells tested were influenced by a single stimulus to the superior laryngeal nerve; 11 were excited and 9 were inhibited. The latency of activation was similar to that following glossopharyngeal stimulation. Of the 22 cells on which the effects of stimuli to both glossopharyngeal and superior laryngeal nerves were recorded, 17 responded to both with the same type of firing pattern whilst opposite effects were observed on 5 cells.

In animals anaesthetized with barbiturate, only one of eight cholinoceptive cells tested could be evoked by superior laryngeal nerve stimulation. This cell showed a pause in firing.

(c) Lingual nerve This input was tested on only 10 neurones confined to anaesthetized preparations. Three were excited, three were inhibited and four were unaffected. The character of the evoked response was similar to that previously described for the glossopharyngeal and superior laryngeal nerves.

(d) Hypoglossal nerve No cells were activated or inhibited by either single stimuli or short tetani to the hypoglossal nerve. Although small field potentials were evoked in this region, these were always referable to the adjacent hypoglossal nucleus.

(4) The effect of acetylcholine antagonists on the synaptic activation of cholinoceptive cells by stimulation of the glossopharyngeal nerve

The pattern of activity following glossopharyngeal nerve stimulation was similar with both cholinoceptive and non-cholinoceptive cells However, in decerebrate rats only, the possibility that acetylcholine mediates this synaptic excitation was tested further on nine cholinoceptive neurones by ejecting DHβE (10-40 nA; eight cells) and atropine (10-50 nA, three cells) whilst studying the probability of firing following nerve stimulation; poststimulus histograms were prepared concurrently with ratemeter records which plotted the responses to ACh and L-glutamate.

On none of the cells tested did DHβE reversibly decrease synaptic as well as ACh excitations. With five cells, higher currents of DHβE than those required to block the action of applied acetylcholine increased the responses both to glossopharyngeal nerve stimulation and to locally administered L-glutamate; this was probably due to a direct excitant effect by DHβE (see section 2b). With the three other cells no significant effect on synaptic responses was observed during the ejection of DHβE.

Of the three cells tested with atropine two showed a reduction in the synaptic response, but this was accompanied by a parallel reduction in the excitations by both L-glutamate and acetylcholine. No effect was seen on the third cell.

Discussion

The observation that cholinoceptive cells are frequently found in the region of the paramedian reticular nucleus parallels the finding of Avanzino *et al.* (1966) in the cat. However, there are no comparable data from any species on cells in the other nuclear groups studied.

In the present study it has been possible to group the ACh responses into two classes, analogous to the type I and II responses seen in cats by Bradley & Dray (1973). Certain technical complications are associated with typing neuronal responses on the basis of latency, since such latency is determined by several experimental parameters, as was originally suggested by del Castillo & Katz (1957). However, we do not think that inconsistencies of retaining and/or ejecting currents can explain the present findings, largely because short latency excitation was associated with a rapid decline at the termination of the ejecting current, and long latency excitation with a prolonged action If the longer latency excitation was caused by excessive retaining currents, then one would expect the excitation to reverse rapidly at the end of the ejection period.

Bradley & Dray (1973) found that responses to ACh in decerebrate animals were predominantly of a fast nature whereas in preparations anaesthetized with barbiturate the proportion of slow responses was increased. No antagonists were used and hence the authors were unable to ascribe the responses to nicotinic or muscarinic receptors. In the present study the 'fast' ACh responses of the decerebrate rats were predominantly DHβE-sensitive, whereas the 'slow' responses of the rats anaesthetized with barbiturate were more often atropine-sensitive. It thus seems possible to classify the action of ACh on brain stem neurones in terms of ACh receptor type. The tests performed with acetyl-β-methylcholine would tend not to substantiate this division of receptor type, but in view of the small number of cells examined and the lack of tests with other cholinomimetics, no conclusions can be drawn other than those based on the actions of the ACh antagonists which were used. Such conclusions do of course depend on the specificity of the actions of these antagonists. In view of their specific action on different cells it does seem likely that they were antagonizing different receptor types; this is in contrast with results on Renshaw cells of rats (Biscoe, Headley & Lodge, 1974).

It seems clear from these results that the nicotinic action of acetylcholine, which was more frequently observed in decerebrate preparations, was reduced by barbiturate anaesthesia. That this was a local action of the barbiturate is likely in view of the action of electrophoretically applied pentobarbitone in rats anaesthetized with urethane. Thus both Bradley & Dray (1973) studying short latency ACh excitation, and Lodge & Martin (unpublished) who specified this excitation as being DHβE-sensitive, found that electrophoretically applied pentobarbitone reduced responses to ACh more than those to L-glutamate or DL-homocysteate.

It is however not clear whether electrophoretically or systemically administered pentobarbitone was altering the nature of the nicotinic receptor to a less effective muscarinic type or whether it was blocking nicotinic receptors thereby revealing muscarinic receptors which would otherwise have gone unnoticed. Coupled with the latter alternative, it is possible that there were two separate populations of cholinoceptive neurones, one with muscarinic and the other with nicotinic receptors, and that in the rat anaesthetized with barbiturate the less common muscarinic neurones were more apparent since the nicotinic receptors of the larger cholinoceptive population had been blocked by the barbiturate. Only this last possibility seems consistent with the finding that DHβE could completely abolish the action of ACh on some neurones in decerebrate preparations and also with the apparent greater ease with which cholinoceptive cells were found in decerebrate preparations.

This differential effect by pentobarbitone is an unusual finding when compared with results obtained by other workers in the cat. In the thalamus of the cat anaesthetized with barbiturate Andersen & Curtis (1964) found that DHβE specifically antagonized the action of acetylcholine whereas atropine did not; the reverse of this, however, was reported by McCance, Phillis, Tebēcis & Westerman (1968). Acetylcholine-sensitive cells of the cerebral cortex have receptors of the muscarinic type regardless of the presence or absence of barbiturates (Krnjević & Phillis, 1963). On the Renshaw cell of the cat, pentobarbitone does not reduce the responses to nicotinic or muscarinic agonists before the response to DLH is also affected (Curtis & Ryall, 1966).

The present study shows that pentobarbitone does have an action on cholinoceptive neurones of the rat paramedian reticular nucleus. In the unanaesthetized cat however, 'fast' ACh responses have not been found in this area (Bradley *et al.*, 1966; Duggan & Game unpublished). Thus any inferences as to the relevance of this effect to barbiturate anaesthesia would be premature.

Controversy exists over possible synaptic inputs to the paramedian reticular nucleus in the cat; the claim that stimulation of the sinus nerve produces short latency activation of cells in this area (Crill & Reis, 1968; Miura & Reis, 1969) has not been confirmed by others (Biscoe & Sampson, 1970; Spyer & Wolstencroft, 1971). The segment of glossopharyngeal nerve stimulated in the present study in the rat was distal to its junction with the sinus nerve. Further, stimulation was usually associated with a rise in blood pressure. Hence, it is unlikely that impulses in fibres of the sinus nerve were responsible for the long latency activation of cells of the paramedian reticular nucleus. Similar neuronal responses were obtained following stimuli to glossopharyngeal, superior laryngeal and lingual nerves but, by comparison, no evidence was found of any synaptic input from the hypoglossal nerve. The present study, whilst demonstrating long latency inputs from these cranial nerves, does not provide any positive evidence as to the transmitters involved in these processes. However, since non-cholinoceptive as well as cholinoceptive neurones are activated in a similar manner, and since DHβE and atropine fail to influence this activation, it is unlikely that acetylcholine mediates the synaptic excitation at the level of the paramedian reticular and perihypoglossal neurones.

We are grateful to Miss E. Thornton and Mr K.W.T. Caddy for technical assistance, to the Wellcome Trust, the Animal Health Trust and the N.H. and M.R.C. of Australia for grants towards recurrent costs and to Merck, Sharpe & Dohme for a gift of dihydro-β-erythroidine. We also wish to thank Professor T.J. Biscoe for advice and encouragement. A.W.D. is a C.J. Martin Fellow, P.M.H. is a Wellcome Trust Research Training Scholar and D.L. is a Wellcome Trust/Animal Health Trust Research Fellow.

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A COMPARISON OF THE EFFECTS OF CYTOSINE ARABINOSIDE AND ADENINE ARABINOSIDE ON SOME ASPECTS OF BRAIN GROWTH AND DEVELOPMENT IN THE RAT

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1 Treatment of pregnant rats with cytosine arabinoside (ara-C, 50 mg/kg, i.p.) at 14 days of gestation severely impaired both prenatal and postnatal whole brain growth in their offspring, although the cerebellum was relatively less affected than whole brain.

2 Rats treated at 5 days of age with ara-C (250 mg/kg, i.p.) showed an impairment in growth of the cerebellum relative to the rest of the brain.

3 Adenine arabinoside (ara-A) treatment, either prenatally or postnatally, had negligible effect on brain growth, even at doses considerably higher than those of ara-C.

4 Adult rats, previously treated with ara-C (50 mg/kg, i.p.) at 14 days of gestation, showed an impairment in discrimination learning when tested in a water T-maze.

5 These results are discussed in relation to the proposed use of ara-C or ara-A as antiviral agents, particularly against intrauterine infection with cytomegalovirus

Introduction

Congenital infection with cytomegalovirus may have serious consequences for brain development (Hanshaw, 1966; McCracken, Shinefield, Cobb, Rausen, Dische & Eichenwald, 1969; Berenberg & Nankervis, 1970), and there are increasing suspicions that, in some cases, microcephaly and mental retardation may result even when the immediate clinical symptoms are negligible (Hanshaw, 1966, Stern, Elek, Booth & Fleck, 1969). Many infants with congenital infection have probably been exposed to the virus during a large part of intrauterine development. There is therefore a quest for suitable antiviral agents to treat the condition during the neonatal period, and perhaps also, during pregnancy.

Cytomegalovirus is a DNA virus of the herpes group whose replication may be prevented by inhibitors of DNA synthesis (Sidwell, Arnett & Brockham, 1970) and in particular by cytosine arabinoside (ara-C). Ara-C is an antimetabolite which acts primarily as an inhibitor of DNA polymerase and has been used, with varying success, to treat cytomegalovirus infection in the neonatal period (Kraybill, Sever, Avery & Movassaghi, 1972; McCracken & Luby, 1972). The toxicity of ara-C is well known from its use in leukaemia, and in a developing organism this drug might in particular affect neuronal multiplication.

It is therefore necessary to assess whether ara-C, in the doses employed therapeutically, has any adverse effect on brain development. Such effects might be most marked if the drug were given during the major period of neurogenesis, 10 to 18 weeks of gestation in man (Dobbing & Sands, 1973) or 14 to 21 days of gestation in the rat (Croskerry, Smith, Shepard & Freeman, 1973).

We have investigated the effects of ara-C, given at either 14 days of gestation or 5 days of postnatal age, on some aspects of brain growth and development in the rat. At 5 days of age the rat brain is in a phase of development comparable with the human brain at term (Dobbing & Sands, 1973). The corresponding effects of adenine arabinoside (ara-A) have also been studied. This substance, probably of lower toxicity than ara-C (Kurtz, Fisken, Kaump & Schardein, 1969), has been suggested (Miller, Dixon, Ehrlich, Sloan & McLean, 1969, Schabel, 1970; Plotkin, 1972) as an alternative antiviral agent.

Methods

Animals

Rats of the Lister black and white hooded strain were fed a good quality diet (Breeding Diet for Rats and Mice, Oxoid Ltd.). Maternal animals had previously reared either one or two litters. The day of mating (day 0) was determined by examination of vaginal smears for sperm.

Drugs

Cytosine arabinoside (1-β-D-arabinofuranosylcytosine; Upjohn Ltd.) was administered as a solution in 0.9% w/v NaCl solution (saline). Adenine arabinoside (9-β-D-arabinofuranosyladenine; Parke, Davis and Co.), which is sparingly soluble in water, was given as a suspension in saline. Control animals received saline only. Drugs and saline were injected intraperitoneally at a dose of 10 ml/kg in both pregnant and postnatal rats, and were always administered at approximately 10 h 00 minutes.

Prenatal treatment

At 14 days of gestation pregnant rats received single injections of saline (controls), ara-C (50 mg/kg), ara-A (50 mg/kg) or ara-A (1 g/kg). All litters were born at either 21 or 22 days of gestation. Irrespective of the length of gestation litters were reduced to 8 animals (equal numbers of each sex) on day 22. At the same time a number of excess newborn were killed from each litter. No offspring of mothers receiving ara-A (1 g/kg) were examined beyond birth. In litters of the other 3 groups, up to 6 rats (2 males, 4 females) were killed at 25 days of age. From each control or ara-C (50 mg/kg) litter 2 male rats were weaned (at 25 days) and finally killed, after T-maze testing when 15 weeks old.

Postnatal treatment – single doses

Litters were reduced to 6 males at birth. At 5 days of postnatal age 2 rats (controls) in each of 6 litters were injected with saline while the remaining 4 rats in each litter were injected once with one of the following: ara-C (50 mg/kg), ara-C (250 mg/kg), ara-A (50 mg/kg) or ara-A (250 mg/kg). All animals were killed when 25 days old.

Postnatal treatment – multiple doses of adenine arabinoside

Litters were reduced to 6 males at birth. On days 3, 4, 5 and 7, two rats (controls) in each of 6 litters were injected with saline, 2 rats received ara-A (250 mg/kg on each day) and 2 rats received ara-A (1 g/kg on each day). All animals were killed when 25 days old.

Chemical assays

Brain DNA was determined according to Zamenhof, Bursztyn, Rich & Zamenhof (1964), and brain protein by the method of Lowry, Rosebrough, Farr & Randall (1951).

Pigmentation

Pigmentation of these black and white hooded rats is affected by inhibitors of DNA synthesis, presumably when administration coincides with the elaboration of melanocytes. A pigmentation index was therefore determined at 25 days of age. This represented the proportion of the back (behind the base of the skull and to halfway down the flanks) which was black. The index was estimated by drawing, on graph paper, the approximate shape of the pigmented areas on a rat-shaped template.

T-maze

Rats aged 15 weeks were tested in a water T-maze as previously described for guinea-pigs (Adlard, Moon & Smart, 1974). The light cycle was 12 h red, beginning at 12 h 00 min, and 12 h white. Rats were required to swim and make a left-right discrimination in order to escape. Escape was possible from one arm only. An entry into a blocked arm, or re-entry into the start arm, constituted an error. Testing was carried out at 09 h 00 min-12 h 00 minutes. Each animal was given 10 trials per day for 6 days. On days 1-4 escape was possible from the same arm (left or right, randomly assigned to each rat). On days 5 and 6 the escape route was reversed and the ability to learn the reversal was examined.

Statistics

Except where stated otherwise mean values were compared by Student's t test.

Results

Prenatal growth after prenatal treatment with cytosine arabinoside or adenine arabinoside

A single dose of ara-C (50 mg/kg) at 14 days of gestation resulted in a birth weight deficit of 14% (Table 1). The brain was relatively more affected, with a weight deficit of 17%, and the brain/body ratio was significantly reduced. Treatment with ara-A, even at 1 g/kg, did not affect prenatal growth.

The brain weight deficit in offspring of ara-C

Table 1 Effect of single intraperitoneal dose of cytosine arabinoside (ara-C) or adenine arabinoside (ara-A) on day 14 of gestation on mean body and brain weight at birth.

	Control	*Ara-C (50 mg/kg)*	*Ara-A (50 mg/kg)*	*Ara-A (1 g/kg)*
Body wt. (g)	5.76 ± 0.79 (78)	4.98 ± 0.45* (60)	5.64 ± 0.64 (67)	5.56 ± 0.70 (68)
Brain wt. (mg)	228 ± 24 (31)	189 ± 20* (14)	236 ± 18 (27)	233 ± 18 (15)
Brain wt./body wt. ratio (%)	4.21 ± 0.26 (31)	3.82 ± 0.31* (14)	4.35 ± 0.40 (27)	4.38 ± 0.46 (15)

Results (mean ± s.d. of the number of animals indicated in parentheses) are based on the following numbers of litters: control, 7; ara-C (50 mg/kg), 6; ara-A (50 mg/kg), 5; ara-A (1 g/kg), 7.
* $P < 0.001$, compared with controls.

treated mothers reflected a deficit in number of brain cells as assessed by total DNA (Table 2). Brain protein concentration was also reduced, but the protein/DNA ratio (Table 2) was normal, suggesting that average cell size was not significantly affected.

Table 2 Effect of a single intraperitoneal dose of cytosine arabinoside (ara-C) on day 14 of gestation on mean brain DNA and protein at birth.

	Control	*Ara-C (50 mg/kg)*
Number of rats	31	14
Total brain DNA (μmol DNA-P)	1.65 ± 0.20	1.17 ± 0.12*
Brain protein (mg/g wet wt.)	67.7 ± 6.9	62.1 ± 5.8†
Brain protein (mg/μmol DNA-P)	9.51 ± 2.28	10.04 ± 1.39

Results (mean ± s.d.) are based on 7 control and 6 ara-C-treated litters.
* $P < 0.001$, † $P < 0.02$, compared with controls.

Postnatal treatment with cytosine arabinoside

A linear regression analysis shows that a single dose of ara-C at 5 days of age significantly reduced cerebellar weight and the ratio between cerebellar weight and brain weight at 25 days (Table 3). Although whole brain weight following the higher dose of ara-C was significantly less than that in controls ($P < 0.02$), this is entirely attributable to the effect on cerebellar weight. There was no effect on body growth. In ara-C treated animals hair growth was delayed by several days, but the extent of pigmentation of the hair was not different from that of the controls (compared with results on animals receiving ara-C prenatally).

Postnatal treatment with adenine arabinoside

A single dose of ara-A at 5 days of age did not affect growth of the body, whole brain or cerebellum to 25 days of age (Table 3). Half of the rats receiving 4 daily doses of ara-A (1 g/kg) failed to thrive and died before 25 days (Table 4). This represented a significant ($P < 0.01$, Fisher test) mortality compared with control or ara-A (4 doses

Table 3 Effect of a single intraperitoneal dose of cytosine arabinoside (ara-C) or adenine arabinoside (ara-A) at 5 days of postnatal age on mean body and brain weight at 25 days of age.

	Control	*Ara-C (50 mg/kg)*	*Ara-C (250 mg/kg)*	*Ara-A (50 mg/kg)*	*Ara-A (250 mg/kg)*
Number of rats	11	6	7	7	6
Body wt. (g)	55.2 ± 10.1	53.3 ± 14.4	48.3 ± 10.4	53.9 ± 12.7	57.2 ± 12.1
Brain wt. (mg)	1387 ± 110	1370 ± 106	1280 ± 111	1371 ± 91	1392 ± 132
'Forebrain' + 'Stem' (mg)	1188 ± 86	1192 ± 88	1128 ± 85	1177 ± 73	1193 ± 100
Cerebellum wt. (mg)	195 ± 24	179 ± 15	152 ± 26	194 ± 22	199 ± 31
Cerebellum wt./whole brain wt. ratio (%)	14.0 ± 1.0	13.0 ± 0.5	11.8 ± 1.1	14.1 ± 0.8	14.2 ± 0.9

Results (mean ±s.d.) are based on 6 litters.
Linear regression analysis of effects of ara-C within this dose range on cerebellum weight and cerebellum weight/whole brain weight ratio shows that both are highly significant ($P < 0.001$).

of 250 mg/kg) animals, none of whom died. There was a decrease (Table 4) in cerebellar weight (10%) in the 4 x 1 g/kg group but this was rather small compared with the reduction (22%) observed after one sixteenth the quantity of ara-C (single dose of 250 mg, Table 3). Postnatal treatment with ara-A affected neither growth of the hair nor its pigmentation.

Postnatal growth after prenatal treatment with cytosine arabinoside or adenine arabinoside

A single dose of ara-C (50 mg/kg) at 14 days of gestation resulted, at 25 days of postnatal age, in a 22% deficit in brain weight, but with no significant effect on body weight (Table 5). Cerebellar weight was affected less than that of whole brain. Prenatal treatment with ara-A (50 mg/kg) had no effect on postnatal brain growth.

Table 4 Effect of daily intraperitoneal doses of adenine arabinoside (ara-A) on postnatal days 3, 4, 5 and 7 on mean body and brain weight at 25 days of age

	Control	*Ara-A (4 x 250mg/kg)*	*Ara-A (4 x 1 g/kg)*
Number of rats	11	12	6
Body wt. (g)	60.1 ± 7.3	60.8 ± 5.9	53.2 ± 4.5*
Brain wt. (mg)	1402 ± 59	1405 ± 43	1361 ± 62
Cerebellum wt. (mg)	199 ± 11	199 ± 11	182 ± 14*
Cerebellum wt./whole brain wt. ratio (%)	14.2 ± 0.5	14.2 ± 0.6	13.4 ± 0.7*

Results (mean ± s.d.) are based on 6 litters.
* $P < 0.05$, compared with controls.

Table 5 Effect of a single intraperitoneal dose of cytosine arabinoside (ara-C) on day 14 of gestation on mean body and brain weights at 25 days of age, and on hair pigmentation.

	Control	*Ara-C (50 mg/kg)*	*Ara-A (50 mg/kg)*
Number of rats	39	23	28
Body wt. (g)	55.1 ± 7.7	51.6 ± 7.7	57.1 ± 3.8
Brain wt. (mg)	1375 ± 67	1069 ± 107*	1385 ± 40
Cerebellum wt. (mg)	189 ± 12	169 ± 19*	196 ± 18
Brain wt./body wt. ratio (%)	2.50 ± 0.27	2.10 ± 0.21*	2.43 ± 0.26
Cerebellum wt./whole brain wt. ratio (%)	13.7 ± 0.5	15.9 ± 1.7*	14.1 ± 0.8
Pigmentation index (%)	30.3 ± 6.0	14.5 ± 4.8*	31.0 ± 3.5

Results (mean ± s.d.) are based on the following numbers of litters. control, 7; ara-C, 6; ara-A, 5.
* $P < 0.001$, compared with controls.

Adult (15-week-old) male rats whose mothers received ara-C (50 mg/kg) prenatally also showed specific microcephaly in that body weight was normal, but brain weight was reduced by 15% (Table 6).

Pigmentation

Ara-C (50 mg/kg) at 14 days of gestation severely reduced the formation of hair pigment in offspring (Table 5). Figure 1 shows two typical control adults compared with two animals of the ara-C group. The latter show normal head pigmentation, but their backs have very little pigment Ara-A had no effect on pigmentation (Table 5).

T-maze

During the first four days of testing in the T-maze the control rats improved progressively in terms of both errors made and number of errorless runs (Figure 2). In contrast, those animals treated prenatally with ara-C did not improve after day 2. Ara-C rats made significantly more errors than controls on days 3 and 4, and fewer errorless runs on day 4 (Figure 2). Over days 1-4, the ara-C group made 80% more total errors than the controls (Table 6). Despite their relatively poor performance on initial learning, ara-C rats did not differ from controls on reversal (days 5 and 6, results not shown). Behavioural testing was not carried out on rats treated prenatally with ara-A, since this treatment had no effect on later brain growth.

Table 6 Effect of a single intraperitoneal dose of cytosine arabinoside (ara-C) on day 14 of gestation on mean body and brain weights and on T-maze errors by 15-week-old male rats.

	Control	*Ara-C (50 mg/kg)*
Number of rats	12	11
Body wt. (g)	304.± 35	294 ± 32
Brain wt. (mg)	1760 ± 56	1497 ± 68*
Brain wt./body wt. ratio (%)	0.584 ± 0.062	0.512 ± 0.045†
Total T-maze errors (days 1-4)	6.1 ± 3.6	10.9 ± 3.9†

Results (mean ± s.d.) are based on 6 litters of each group.
* $P < 0.001$, † $P < 0.01$, compared with controls.

Figure 1 Hair pigmentation in 15-week-old male rats. Each rat was from a different litter. Animals at far left and far right were controls The two centre rats were offspring of mothers receiving cytosine arabinoside (50 mg/kg) on day 14 of pregnancy

Discussion

In attempts to treat cytomegalovirus infection in infants ara-C has been administered over about 10 days in a total dose of approximately 50 mg/kg (Plotkin, 1972). This is a similar regime to that used in leukaemia and; in the present work, the same dose was given, but as a single injection. Species extrapolations are particularly difficult in the present context because of the very rapid rate of brain development in the rat. At this stage of brain development, a day in the life of a rat is equivalent to more than a month in the life of a human child. Thus it may be that even a single injection in the rat exposes the developing brain to the drug for a relatively longer period than multiple daily dose in man. For these reasons the present results are probable pointers to the qualitative, but not the quantitative, effects of ara-C on human brain development.

Ara-C exerted the much more severe effect on brain growth when given prenatally, at a time of initiation of rapid neuronal multiplication (Croskerry *et al.*, 1973). The deficit in brain cells at birth must have been largely of neurones, since prenatal brain cell division in this species is predominantly neuronal. For this same reason it was also probably permanent. Similarly glial cell multiplication in the rat brain is almost exclusively postnatal and therefore it is this process which must have been inhibited after ara-C treatment at 5 postnatal days of age. This had no effect on whole brain growth which could not be accounted for by the effect on the cerebellum.

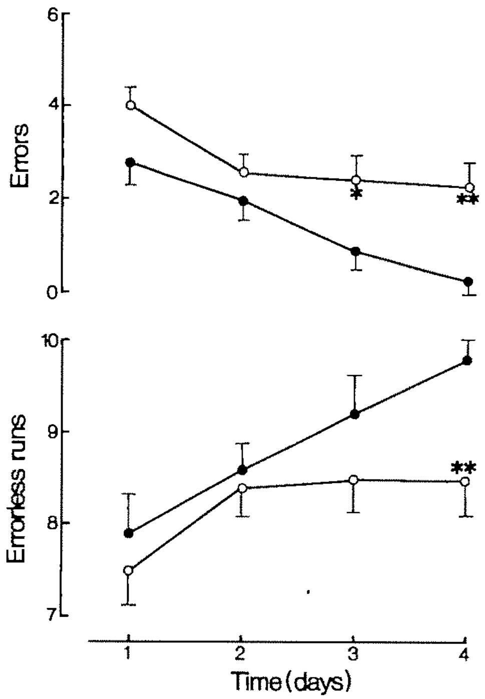

Figure 2 T-maze performance by adult male rats. Results represent means (± s.e. mean) of 12 control rats (●), and of 11 cytosine arabinoside (ara-C) rats (○) whose mothers received ara-C (50 mg/kg) on day 14 of gestation. Each animal was given 10 runs on each day of testing.
* $P < 0.05$, ** $P < 0.01$, compared with controls.

From this it follows that neuronal (in contrast to total 'brain cell') number may be an important determinant of mature brain size, and the results on cerebellar growth support this concept. Neurogenesis in the rat cerebellum, unlike that in the rest of the brain, is primarily a postnatal event. Correspondingly, cerebellar growth was most retarded, both absolutely and relative to whole brain, when ara-C was given postnatally. The effects of ara-A, ara-C and hypoxanthine arabinoside on the developing cerebellum have been reported recently in considerable detail (Fishaut, Connor & Lampert, 1974).

The effects of prenatal ara-C in many ways resembled those observed after prenatal treatment with hydroxyurea (Adlard & Dobbing, 1975). Neither ara-C nor hydroxyurea produced any major malformations, but both treatments reduced the extent of hair pigmentation, probably through interference with melanoblast division and/or migration. Ara-C seemed to produce a more specific effect on the brain than hydroxyurea in that microcephaly (low brain/body ratio) was observed as early as the time of birth. Moreover, the stunting of postnatal bodily growth, observed after hydroxyurea, was not found in rats treated with ara-C.

Ara-A was not toxic to prenatal brain development, even at 20 times that dose of ara-C which produced a 29% deficit in brain DNA at birth (Tables 1 and 2). In addition, prenatal ara-A did not affect postnatal brain growth. Postnatal treatment with ara-A had a small effect on cerebellar growth, but only at very high and multiple doses. Since these doses approached the LD_{50} observed in adult mice (Kurtz *et al*, 1969) and were associated with a high mortality and impaired bodily growth, the effect on the cerebellum may well have resulted from general debility rather than from a specific influence on the brain.

The present results suggest that ara-C may be unsuitable as an antiviral agent for administration at a time of major brain development. This would apply particularly if the drug were given in early or mid-pregnancy where its effects on brain growth might be at least as severe as those of cytomegalovirus infection itself. Caution is particularly indicated by the apparent learning impairment (T-maze performance) resulting from prenatal treatment with ara-C. Growth of the developing brain was relatively resistant to ara-A, and this drug, which seems to be of comparable potency to ara-C in its action against DNA viruses (Miller *et al*, 1969), may be more acceptable as a therapeutic agent. Nevertheless ara-A has a known teratogenic potential in developing rabbits (though not in monkeys) at doses greater than 3 mg/kg, (personal communication by S.M. Kurtz). The recommended dose of ara-A for the treatment of cytomegalovirus in humans is 15-20 mg/kg daily by slow intravenous infusion for at least 10 days. Even ara-A should therefore probably be avoided during pregnancy.

We thank Dr J.A.L. Gorringe of Parke, Davis and Co. for advice and a gift of adenine arabinoside. This work was supported by the Medical Research Council and the National Fund for Research into Crippling Diseases.

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(Received October 21, 1974.
Revised January 31, 1975)

Br. J. Pharmac. (1975), 54, 41-48

RENAL TUBULAR EXCRETION OF TRIETHYLCHOLINE (TEC) IN THE CHICKEN: ENHANCEMENT AND INHIBITION OF RENAL EXCRETION OF CHOLINE AND ACETYLCHOLINE BY TEC

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1 [^{3}H]-triethylcholine (TEC) was actively transported by the renal tubule of the chicken at a rate 85% that of simultaneously administered *p*-aminohippuric acid (PAH).

2 TEC was demonstrated to be transported by the organic cation transport system in the kidney through inhibition with quinine and the bio-cation choline.

3 When the infusion of TEC was increased to 2×10^{-6} mol kg^{-1} min^{-1} reaching the infused kidney, the transport of [^{3}H]-TEC was inhibited, suggesting that an excretory transport maximum for TEC in the renal tubules had been reached.

4 The excretion of both choline and acetylcholine was enhanced by TEC loads as low as 1×10^{-18} mol kg^{-1} min^{-1}. Enhancement continued as TEC infusion was increased up to approximately 1×10^{-7} mol kg^{-1} min^{-1} at which point this enhancement was converted to inhibition.

5 Possible mechanisms for the biphasic effect of TEC on organic cation transport are discussed.

Introduction

Choline is one of the substrates for acetylcholine synthesis at cholinergic nerve endings and these nerve terminals are known to possess a transport process for the translocation of the choline from the extracellular fluid to the intraneuronal sites of its acetylation. (Potter, 1968; Marchbanks, 1968; Yamamura & Snyder, 1972; Hubbard, 1973). The plasma concentration of choline has been the subject of a number of investigations (Hunt, 1915; Bligh, 1952; 1953) and homeostatic mechanisms maintain a stable plasma choline level (Gardiner & Paton, 1972; Acara & Rennick, 1973).

The proximal renal tubule can actively transport organic cations from the blood to the urine and choline and acetylcholine are examples of bio-cations that are transported by the renal tubule (Rennick, 1958; Acara & Rennick, 1972a,b).

The present experiments were performed to compare the analogue of choline, triethylcholine (triethyl 2-hydroxyethyl ammonium, TEC) with choline in respect to renal transport and metabolism. TEC has been shown to produce neuropharmacological effects at cholinergic nerve terminals which suggest an interaction with endogenous choline (Bowman, Hemsworth & Rand, 1967). The transport of TEC across the renal tubule has not previously been explored and the present experiments were designed to investigate the effect of TEC on the renal tubular transport of choline and acetylcholine.

The pharmacological activity of TEC is similar to that of the hemicholinium, HC-3 (Bowman & Rand, 1961; Bowman *et al.*, 1967). HC-3 has been shown to enhance the excretion of choline in the chicken at an infusion rate of only 1×10^{-22} mol kg^{-1} min^{-1} (Acara, Kowalski & Rennick, 1973). In the present experiments TEC was also shown to enhance choline excretion.

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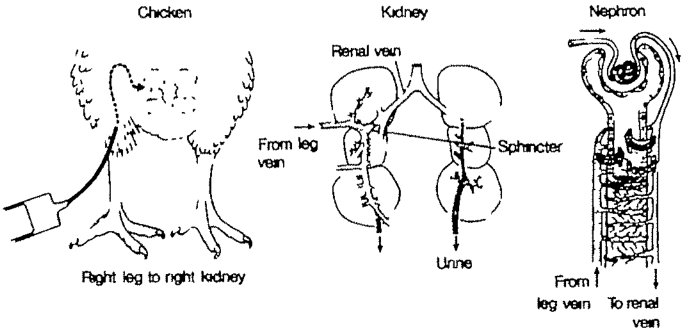

Figure 1 Diagram of renal portal circulation in chicken kidney Infusion of transportable substrates into right leg flows to the right kidney and enters the portal circulation to bathe only the tubules of the right kidney (From Acara, Kowalski & Rennick, (1973), reproduced by permission of the *Journal of Pharmacology and Experimental Therapeutics*).

Methods

Sperber technique for the determination of active transport in chickens

The Sperber technique for the study of renal tubular transport (Sperber, 1948; Lindahl & Sperber, 1958) takes advantage of a separate renal portal circulation in the chicken, accessible through a leg vein (Figure 1). Urine is collected separately from each ureter and the excretion per minute of substrate from the infused and control kidney is measured. Data are expressed as apparent tubular excretion fraction (ATEF) and are calculated as follows:

$$\mathrm{Exc}_{(\mathrm{Infused})} - \mathrm{Exc}_{(\mathrm{Control})}/\mathrm{Amount\ infused}.$$

The ATEF thus represents the proportion of the infused substrate actively excreted by the tubules of the infused kidney. In the present experiments the ATEF is expressed as a percentage. To show active transport, an ATEF of greater than 8% is necessary since this percentage could result from diffusion. The ATEF of *p*-aminohippuric acid (PAH) is a measure of the amount of infusion which reaches the infused kidney since PAH is completely extracted by the renal tubules. Transport efficiency (TE) expresses the ratio, ATEF substrate/ATEF (PAH). The TE relates the amount of compound that was transported by the renal tubule to that which reached the kidney.

Collection of urine

Chickens weighing between 2-3 kg were used without anaesthesia and the experimental procedure was that described previously (Acara & Rennick, 1972a). To maintain an adequate urine flow the chickens were hydrated with 60 ml of water by crop tube at the start of each experiment. Additional loads of 50 ml of water were given every 30 or 40 minutes. During the saline (0.9% w/v NaCl solution) infusion, the ureters were exposed in the cloaca, local anaesthetic applied, and a plastic tube for urine collection was sewn over each ureter. Urine was collected for a period of 10 min and unless otherwise indicated all data represent the mean of three 10 min urine collections.

Infusion solutions

It has been shown previously that choline is actively transported by the renal tubule only at infusion rates greater than 1×10^{-6} mol kg^{-1} min^{-1} reaching the kidney (Acara & Rennick, 1972a). At infusion rates lower than this, choline was extensively metabolized by the renal tubule. A predominant metabolite was betaine which was not transported by the renal tubule. For this reason, the choline infusion rate was kept high for all experiments at 1×10^{-5} mol/minute. A tracer amount of choline-[methyl-^{14}C] was added to the choline infusion solution. Acetyl-[1-^{14}C] choline was infused at a rate of 1×10^{-10} mol kg^{-1} min^{-1}. All infusion rates were factored by the simultaneous PAH excretion to give values that represent the amount of the infused TEC actually reaching the infused kidney.

Analysis of urine

Aliquots of collected urine were added to Bray's (1960) scintillation solution and counted at double label settings for 10 min in a Nuclear-Chicago scintillation spectrometer. Correction was made for the crossover of ^{14}C into the tritium channel. Where large concentrations of TEC iodide were infused and thus entered the urine, the colour quenched the ct/min reading in the spectrometer when Bray's solution was used The substitution of Aquasol (New England Nuclear Corp , Boston, Mass.) was made to prevent any quenching. The ^{14}C-label, as either choline-[methyl-^{14}C] or acetyl-[1-^{14}C] choline, was not isolated and identified in urine collections but was assumed to represent the infused compound. Previous studies had given evidence for transport of the intact molecule of choline and acetylcholine (Acara & Rennick, 1972a,b).

Materials

p-Aminohippuric-[1-^{14}C] acid ([^{14}C]-PAH) 2 52 mCi/mmol and *p*-aminohippuric-[glycyl-2-^{3}H] acid ([^{3}H]-PAH) 205.8 mCi/mmol were obtained from New England Nuclear Corporation, Boston, Mass. Acetyl-[1-^{14}C] choline chloride, 11.7 mCi/mmol and choline chloride-[methyl-^{14}C], 54 mCi/mmol were obtained from Amersham/Searle Corporation, Des Plaines, Illinois. One source of TEC was tritium labelled by a catalytic exchange process by New England Nuclear Corp. and was purified by thin layer chromatography by the method of Hemsworth & Bosmann (1971). The purified [^{3}H]-TEC had a specific activity of 37.1 mCi/mmol. In some experiments [^{3}H]-TEC, 25 mCi/mmol was used as purchased from New England Nuclear Corp. TEC chloride and TEC iodide were used as carrier

Results

Renal tubular transport of TEC

The ipsilateral infusion of [^{3}H]-TEC into a leg vein at rates of 1 x 10^{-10} to 2 x 10^{-9} mol kg^{-1} min^{-1} into the peritubular blood of the chicken kidney resulted in active tubular transport of TEC. The apparent tubular excretion fraction (ATEF) for TEC was 52% (Table 1) illustrating that active tubular excretion of TEC does occur. The ATEF of PAH of 60.8% (Table 1) represents the proportion of the infused PAH that reached the renal tubules of the infused kidney. If the ATEF of TEC is compared to the ATEF of PAH, the resulting value, the transport efficiency (TE) for TEC of 0.85 ± 0.07 indicates tubular excretion of 85% of that TEC reaching the infused kidney. This percentage value gives a clearer indication of the ability of the tubule cells to transport TEC and demonstrates effective tubular transport.

Inhibition of TEC transport by cationic competitors

One of the criteria used commonly to detect active tubular transport is to measure the inhibitory effect of substances that compete for the same transport pathway, in this case, organic cations. The active transport of TEC was challenged by the organic cation quinine, which at a concentration of 2 x 10^{-7} mol kg^{-1} min^{-1} produced greater than 50% inhibition of TEC transport. The effect of the cation choline on the transport of TEC was also observed and in two experiments 2 x 10^{-6} mol kg^{-1} min^{-1} choline produced 20% inhibition of the transport of TEC. In five experiments, increasing loads of unlabelled TEC were added during a constant infusion of [^{3}H]-TEC (from 1 x 10^{-10} to 2 x 10^{-9} mol kg^{-1} min^{-1}) (Figure 2). As the TEC load was increased, [^{3}H]-TEC transport was inhibited. Loads of TEC around 2 x 10^{-6} mol kg^{-1}

Table 1 Renal tubular transport of triethylcholine (TEC) by the chicken kidney by means of the Sperber technique

	Apparent tubular excretion fraction (ATEF[2] as %)		*Transport efficiency (TE)*
	[^{3}H]-TEC[1]	*[^{14}C]-PAH[1]*	$\frac{[^{3}H]\text{-TEC, ATEF}}{[^{14}C]\text{-PAH, ATEF}}$
	62	66	0 94
	53	66	0.80
	47	58	0.81
	52	53	0.98
	52	66	0.79
	56	60	0.93
	32	37	0.86
	42	53	0.79
	73	82	0.89
	40	54	0.74
	68	79	0.86
	47	55	0.85
Mean	52.00	60.75	0.85
±s.e.	±11.70	±12.21	±0.07

1. Infusion rates. [^{14}C]-PAH at 1.8 x 10^{-10} mol kg^{-1} min^{-1}, [^{3}H]-TEC from 1 x 10^{-10} to 2 x 10^{-9} mol kg^{-1} min^{-1}.

2. $$\text{ATEF} = \frac{\text{Exc}_{\text{infused kidney}} - \text{Exc}_{\text{control kidney}}}{\text{Rate of infusion}}$$

The data on each horizontal line represent an individual chicken experiment.

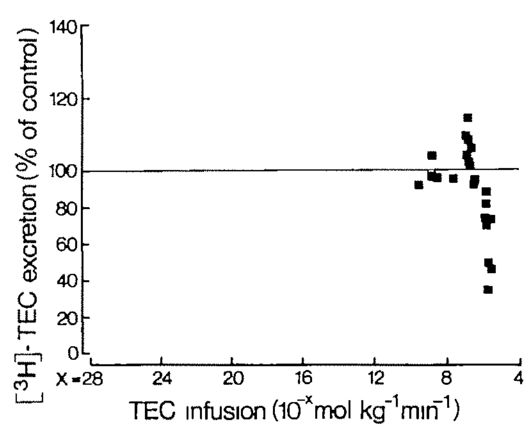

Figure 2 The effects of infusion of triethylcholine (TEC) at increasing concentrations on the renal tubular excretion of [^{3}H]-TEC. The [^{3}H]-TEC excretion is expressed as % of control excretion rate. [^{3}H]-TEC infusion rate from 1 x 10^{-10} to 2 x 10^{-9} mol kg^{-1} min^{-1}. All infusion rates were factored by simultaneous *p*-aminohippuric acid excretion to give actual amount reaching infused kidney *n* = 5.

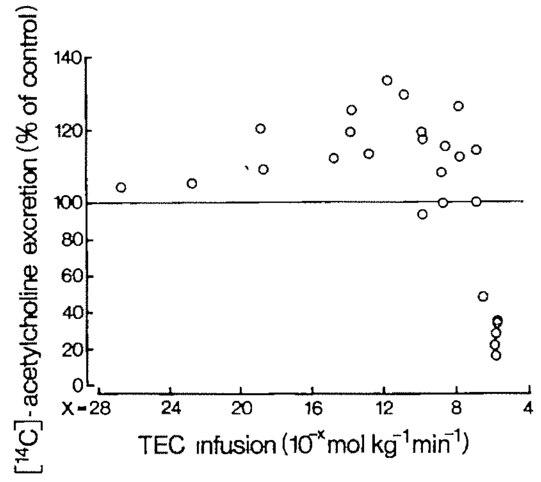

Figure 3 The effect of infusion of triethylcholine (TEC) at increasing rates on the renal tubular excretion of [^{14}C]-acetylcholine. The [^{14}C]-acetylcholine infusion rate was 7.1 x 10^{-12} to 8.7 x 10^{-11} mol kg^{-1} min^{-1} *n* = 5. Conditions as in Figure 2.

min^{-1} reaching the infused kidney, produced a 70% inhibition of [^{3}H]-TEC transport.

Enhancement of transport of choline and acetylcholine by very low infusion loads of TEC and inhibition by high loads of TEC

The Sperber technique has been used by Acara & Rennick (1972a,b) to demonstrate that both choline and acetylcholine are actively transported by the renal tubule in the chicken. Table 2 demonstrates the effects of TEC infusions from 1 x 10^{-18} to 1 x 10^{-5} mol kg^{-1} min^{-1} reaching the infused kidney.

At a concentration of TEC of 1 x 10^{-18} mol kg^{-1} min^{-1} reaching the infused kidney, choline excretion was potentiated 17%. This enhancement of choline excretion persisted with TEC loads up

Table 2 Effect of triethylcholine (TEC) on the tubular transport of [^{14}C]-choline in the chicken kidney measured by the Sperber technique

	ATEF %[2]		*TE*	*% of control*
	[^{3}H]-PAH[1]	*[^{14}C]-choline*	*ATEF [^{14}C]-choline / ATEF [^{3}H]-PAH*	
Control	67	24	0.36	100
During infusion of TEC (mol kg^{-1} min^{-1})				
1 x 10^{-18}	56	23	0.42	117
1 x 10^{-13}	56	24	0.43	120
1 x 10^{-10}	65	28	0.42	117
5 x 10^{-8}	69	24	0.35	85
1 x 10^{-7}	68	24	0.36	88
2.7 x 10^{-6}	60	11	0.19	47
5.5 x 10^{-6}	64	6.4	0.099	24
1 x 10^{-5}	58	3.2	0.056	14

1. Infusion throughout of [^{3}H]-PAH at 2.9 x 10^{-11} mol kg^{-1} min^{-1} and [^{14}C]-choline plus unlabelled choline at 2.8 x 10^{-6} mol kg^{-1} min^{-1} reaching the infused kidney.
2. As in Table 1.

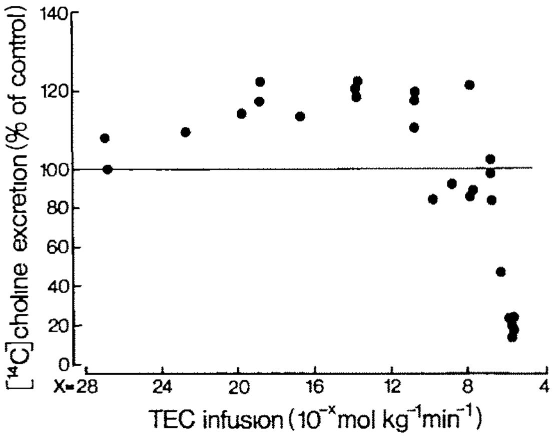

Figure 4 The effects of infusion of triethylcholine (TEC) at increasing rates on the tubular excretion of [^{14}C]-choline. The choline infusion rate was 2.3×10^{-6} to 3.2×10^{-6} mol kg^{-1} min^{-1} All other conditions as in Figure 2. $n = 5$.

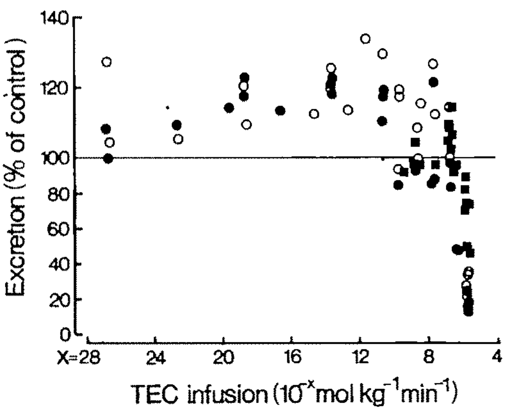

Figure 5 A comparison of the data from [^{14}C]-choline (●), [^{14}C]-acetylcholine (○) and [^{3}H]-triethylcholine (TEC) (■) excretion in the presence of increasing infusions of TEC. Infusion rates as in Figures 2, 3 and 4.

to 5×10^{-8} mol kg^{-1} min^{-1}, when a 15% inhibition of choline excretion occurred. Concentrations of TEC greater than 5×10^{-8} mol kg^{-1} min^{-1} produced even more inhibition of choline excretion, an 86% inhibition being observed in the presence of TEC 1×10^{-5} mol kg^{-1} min^{-1}.

Table 3 shows similar results on the effect of TEC on the transport of [^{14}C]-acetylcholine. TEC perfused into the leg vein to reach the kidney at a concentration of 3×10^{-14} mol kg^{-1} min^{-1} produced a 25% enhancement of acetylcholine excretion. This enhancement of acetylcholine excretion continued as the infusion load of TEC was increased up to 2.5×10^{-8} mol kg^{-1} min^{-1}. At a higher TEC load the enhancement decreased and at a TEC infusion rate of 2×10^{-6} mol kg^{-1} min^{-1}, a 73% inhibition of acetylcholine transport was produced.

Figures 3 and 4 show the results of a number of experiments of the same design as those shown in

Table 3 Effect of triethylcholine (TEC) on the tubular transport of the ^{14}C-label associated with acetylcholine in the chicken kidney measured by the Sperber technique

	*ATEF %*²		*TE*	*% of control*
	*[^{3}H]-PAH*¹	*[^{14}C]-acetyl-1 choline*	*ATEF [^{14}C]-ACh / ATEF [^{3}H]-PAH*	
Control	69	30	0.44	100
During infusion of TEC (mol kg^{-1} min^{-1})				
3×10^{-14}	74	40	0.55	125
3×10^{-12}	72	42	0.58	132
3×10^{-10}	64	36	0.56	127
2.5×10^{-8}	61	34	0.55	125
2×10^{-7}	52	26	0.50	114
2×10^{-6}	47	6	0.12	27

1. Infusion throughout of [^{3}H]-PAH at 0.9×10^{-11} mol kg^{-1} min^{-1} and [^{14}C]-acetylcholine at 3.9×10^{-11} mol kg^{-1} min^{-1}.
2. As in Table 1.

Tables 2 and 3. The excretion of [^{14}C]-acetylcholine (Figure 3) and [^{14}C]-choline (Figure 4) in the presence of varying infusion rates of TEC are plotted as a percentage of the control excretion. Infusion rates of TEC as low as 1×10^{-18} mol kg^{-1} min^{-1} reaching the infused kidney were also shown to enhance the excretion of choline and acetylcholine. The biphasic effect of enhancement of excretion of acetylcholine and choline at low loads of TEC and inhibitions at high loads of TEC is apparent from these experiments.

When the data from the choline, acetylcholine and TEC experiments are plotted together (Figure 5) it can be seen that the effects of TEC are quantitatively similar on the transport of all three of these organic cations.

Discussion

TEC was found to be actively transported across the renal tubule in the chicken kidney *in vivo* and the transport of TEC was found to be blocked specifically by the cationic competitor quinine. The rate of TEC transport, expressed as transport efficiency (TE), 0.85 at an infusion rate of TEC of less than 10^{-9} mol kg^{-1} min^{-1} was greater than that for the maximum TE of choline, 0.30 at an infusion rate of 10^{-5} mol/minute. It is possible that TEC is transported more rapidly than choline because it may not be metabolized to any appreciable extent by the kidney whereas choline is metabolized by the kidney to betaine (Acara & Rennick, 1972a). Choline oxidase is the enzyme present in the kidney tubule which metabolizes choline to betaine and this metabolite is not actively transported. Acetylcholine is another renal metabolite of choline which appears in the urine during choline infusion (Acara & Rennick, 1972b). Acetylcholine is actively transported by the renal tubule during acetylcholine infusion.

TEC is not a substrate for choline oxidase (Wells, 1954) and therefore it is probable that TEC is not oxidized to the corresponding aldehyde in the kidney and excreted in this form. However, TEC is acetylated by choline acetyltransferase, the enzyme responsible for acetylcholine synthesis, although the apparent *Km* for TEC acetylation is much greater than the apparent Michaelis-Menten constant for choline acetylation (Hemsworth & Smith, 1970). It is possible therefore that some of the TEC in the plasma of the chicken after intravenous infusion may be converted to the acetylated form of TEC and excreted in this form. It is not known whether acetyl TEC is actively transported by the kidney tubule. The present results show that [^{3}H]-TEC is rapidly excreted and it is suggested that the major portion of this excreted material is in a non-metabolized form.

At high concentrations TEC inhibits choline and acetylcholine transport through the renal tubule and these effects are similar to those observed on the inhibition of [^{14}C]-choline uptake into synaptosomes and synaptic vesicles (Hemsworth, Darmer & Bosmann, 1971). It is of interest that Acara *et al.* (1973) could find no evidence *in vivo* for inhibition by HC-3 of choline transport across the renal tubule of the unanaesthetized chicken. The unanaesthetized chicken could not tolerate HC-3 at infusion rates greater than 1×10^{-7} mol/min (Acara *et al.*, 1973) and subsequent experiments with the isolated perfused rat kidney demonstrated that HC-3 was an effective inhibitor of renal tubular transport of choline (Trimb~~le,~~ Acara & Rennick, 1974).

HC-3 is generally considered to be the most potent of the drugs which inhibit choline transport. However, although TEC possesses a more marked ganglion blocking action than HC-3 (Bowman & Rand, 1961) the respiratory depression caused by TEC is less than that occurring with HC-3. TEC is therefore less toxic than HC-3 and would appear to be the better choice to investigate choline transport *in vivo*.

The present experiments illustrate that very small loads of TEC enhance the excretion of choline or acetylcholine. As the load of TEC was increased beyond 5×10^{-7} mol kg^{-1} min^{-1} reaching the kidney, a reversal of the response was seen and the excretion of both choline and acetylcholine was inhibited, suggesting a competitive inhibition of tubular transport of these cations by TEC. This represents a biphasic response of the tubular mechanisms. Similar enhancing effects on choline and acetylcholine excretion were seen with HC-3 at similarly low loads (Acara *et al.*, 1973). A likely explanation for the enhancement of excretion of choline or acetylcholine caused by low loads of HC-3 or TEC may relate to the proposed bidirectional transport of choline. In a manner analogous to uric acid transport, choline may be transported actively in both the direction of excretion and reabsorption (Acara & Rennick, 1973). The two directions of transport probably occur simultaneously and the rate of transport in the two directions and the tubular transport maxima (Tm) may be unequal. One direction of the transport may be sensitive to inhibition by lower concentrations of the cationic competitors. In the case of HC-3 and TEC, the reabsorptive transport may be the more sensitive to the low concentrations and hence the net excretion of choline would be enhanced. When high loads of TEC are offered, both reabsorption and excretory transports may be blocked, resulting in a reduced excretion of choline.

HC-3 also does not possess a very potent anticholinesterase action (Domino, Shellenberger & Frappier, 1968; Hemsworth, 1971) again suggesting that anticholinesterase activity does not play any great role in the potentiation of acetylcholine transport by both TEC and HC-3 Both TEC and HC-3, at low concentrations, have previously been reported to enhance the uptake of choline into synaptosomes and synaptic vesicles *in vitro* whereas at higher concentrations of these choline analogues the uptake of choline was inhibited (Hemsworth *et al.*, 1971). It was suggested that both TEC and HC-3 might themselves be transported across the synaptosomal membrane and it is of interest that the present results show that TEC can be transported across the renal tubule by a choline like transport process. It is also of interest that Gomez, Domino & Sellinger (1969) showed that HC-3 caused an increase in choline uptake into dog caudate nucleus *in vivo*. Also Sellinger, Domino, Haarstad & Mohrman (1969) and Rodriguez de Lores Arnaiz, Zieher & de Robertis (1970) showed that HC-3 can be taken up into synaptosomes and synaptic vesicles *in vitro*. It is apparent therefore that both TEC and HC-3 can be transported by the choline transport system. In this respect Hemsworth & Bosmann (1971) have shown that [^{3}H]-TEC is taken up into guinea-pig synaptosomes and synaptic vesicles *in vitro*.

Other workers have demonstrated both enhancement and inhibition of transport by different concentrations of drugs. Holm (1971; 1972) investigated the uptake of some quaternary ammonium compounds and showed that the transport of decamethonium into mouse kidney slices was inhibited by high concentrations of carbamoylcholine, choline, neostigmine and tetraethylammonium, whereas lower concentrations of these drugs potentiated the uptake of decamethonium.

Levine, Oxender & Stein (1965) and Heinz & Walsh (1958) have reported on studies concerning the acceleration of the transport of various hexoses and amino acids. The enhancement of choline and acetylcholine by TEC could be an example of accelerative exchange diffusion or could be due to substrate facilitated carrier transport, however, it seems likely that the carrier mechanism for both choline and acetylcholine and for TEC involves a common quaternary nitrogen carrier mechanism.

The enhancement of excretion of choline and acetylcholine *in vivo* by these small loads of TEC in the present Sperber chicken experiments may result from either an increase in tubular excretion or an inhibition of tubular reabsorption. At the same time the small loads of TEC may alter the intracellular metabolism and disposition of choline and acetylcholine.

The inhibition of excretion of choline and acetylcholine produced by high loads of TEC is probably a result of competition by the TEC for the common organic base carrier which transports choline and acetylcholine across the renal tubule in the direction of excretion.

This work was supported by USPHS Grants AM 10420, HL 14092, F03 AM 52985-01, and United Health Foundation Fellowship FTF-12-UB-71

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(Received July 25, 1974.
Revised September 6, 1974.)

Br. J. Pharmac. (1975), 54, 49-53

MODULATION BY ACETYLCHOLINE OF ADRENERGIC TRANSMISSION IN THE RABBIT EAR ARTERY

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1 Low concentrations of acetylcholine (4×10^{-11} and 1×10^{-10} M) increase the vasoconstrictor response of the isolated ear artery of the rabbit to stimulation of the periarterial sympathetic nerves. Higher concentrations (4×10^{-8} M and greater) decrease the response.

2 Low concentrations of acetylcholine (1×10^{-11} and 1×10^{-10} M) increase the stimulation-induced efflux of radioactivity from artery segments previously incubated with [^{3}H]-noradrenaline. Higher concentrations (3×10^{-8} M and greater) decrease the efflux.

3 Neither atropine nor hexamethonium affects the facilitatory action of low concentrations of acetylcholine on adrenergic transmission in the rabbit ear artery.

4 Atropine antagonizes the inhibitory effect of higher concentrations of acetylcholine on adrenergic transmission.

Introduction

Acetylcholine and other cholinomimetic drugs modify the responses to sympathetic nerve stimulation in isolated artery preparations, depending upon the drug used, the concentration and the frequency of stimulation, the responses may be enhanced or reduced (Malik & Ling, 1969; Rand & Varma, 1970). High concentrations of acetylcholine reduce or abolish the vasoconstrictor responses to sympathetic nerve stimulation in the perfused mesenteric artery of the rat (Malik & Ling, 1969) and the perfused ear artery of the rabbit (Rand & Varma, 1970; Steinsland, Furchgott & Kirkepar, 1973), and this effect is blocked by atropine. However, low concentrations of acetylcholine increase the responses to sympathetic nerve stimulation in these two preparations. It was suggested that the changes in response to sympathetic nerve stimulation produced by acetylcholine were due to changes in release of transmitter. Having developed a method of studying transmitter release in the isolated perfused ear artery of the rabbit (Allen, Rand & Story, 1973a), we set out to test this postulate directly. A preliminary account of some of the findings has been given to the British Pharmacological Society (Allen, Glover, Rand & Story, 1972).

Methods

Vasoconstrictor responses

Rabbits of either sex weighing 2-4 kg were killed by cervical dislocation. The central artery of each ear was cannulated and set up as described by de la Lande & Rand (1965). The preparations were perfused at 6 ml/min with Krebs-Henseleit solution. Perfusion pressure was measured with a Statham P23Db pressure transducer and recorded on a Rikadenki potentiometric recorder, calibrated from 0 to 200 mmHg. Periarterial nerve stimulation was delivered through concentric platinum bipolar electrodes using square wave pulses of 1 ms duration and supramaximal voltage (60-80 V) at a frequency of 2 Hz in trains of 10 s every 2 minutes. Solutions of acetylcholine were infused, by means of a Palmer slow infusion pump at rates of 0.05-0.2 ml/min; infusion of drug-free Krebs-Henseleit solution had no effect on basal pressure or responsiveness of the artery as determined in control experiments.

Experiments with tracer noradrenaline

Segments of the central artery of the rabbit ear were incubated with [^{3}H]-(−)-noradrenaline (1.72 μM; 10 μCi/ml) and then set up for perfusion-superfusion with Krebs-Henseleit solution as described by Allen *et al.* (1973a): the segment was perfused through the lumen and the effluent then superfused the adventitial surface. The flow rate was maintained at 4 ml/min and the perfusion pressure was monitored. Samples of the perfusate-superfusate were collected at 3 min periods for measurement of the efflux of radioactivity from the artery segment. The adventitial sympathetic nerves were stimulated with trains of pulses at 5 Hz for 30 s periods. The first period of stimulation was given 50 min after the period of incubation with [^{3}H]-noradrenaline. The second period was given 30 min after the first. The resting efflux of radioactivity was taken as the efflux in the 3 min period immediately preceding each period in which stimulation was applied. The stimulation-induced efflux was calculated by subtracting the resting efflux from the efflux during the stimulation period. In some experiments the effect of acetylcholine on the resting efflux was monitored by collecting samples of the perfusate-superfusate immediately before, during and after the infusion of acetylcholine. The total radioactivity of samples was calculated in disintegrations per minute (d/min) per collection sample. Corrections for counting efficiency were made using an internal reference standard ([^{3}H]-*n*-hexadecane). Drug solutions were perfused into the perfusion fluid, using a Braun slow injection apparatus.

The Krebs-Henseleit solution was of the following composition (mM) NaCl, 118; KCl, 4.7, $NaHCO_3$, 25; $MgSO_4$, 0.45; KH_2PO_4, 1.03; $CaCl_2$, 2.5; and D-glucose, 11.1. Disodium ethylenediamine tetraacetic acid (EDTA) was added to the Krebs-Henseleit solution to retard oxidation of noradrenaline.

Radiochemicals and drugs

Tritiated *laevo*-noradrenaline ([7-^{3}H]-(−)-noradrenaline acetate) with a specific activity of 5.8 Ci/mmol was obtained from the Radiochemical Centre, Amersham, and was stored at −30° C.

The following drugs were used: acetylcholine perchlorate (B.D.H.); atropine sulphate (David G. Bull Laboratories, Melbourne); hexamethonium bromide (May & Baker), neostigmine methylsulphate (Prostigmin, Roche). All solutions of drugs were freshly prepared in distilled water.

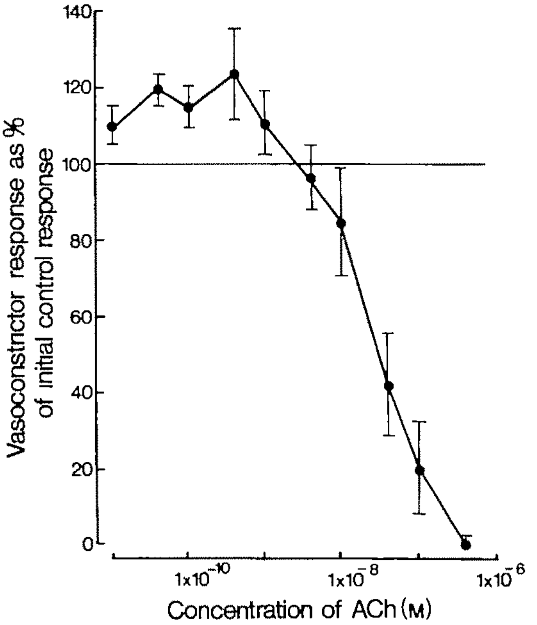

Figure 1 Effects of acetylcholine (ACh) on vasoconstrictor responses to sympathetic nerve stimulation (2 Hz, 10 s) of rabbit isolated ear artery. The rise in perfusion pressure with stimulation in the presence of each concentration of acetylcholine was expressed as a percentage of the initial control value. The points represent means and the vertical bars the standard errors of means of 5-7 experiments.

Results

Effects of acetylcholine on vasoconstrictor responses

After the artery segments were set up and basal pressure was steady (usually around 15 mmHg), stimulation at 2 min intervals was begun. The rise in perfusion pressure with stimulation at 2 Hz for 10 s was about 80 mmHg. At least 6 constant responses were obtained to provide the mean control value, then acetylcholine was infused with step-wise increases in concentrations from 1×10^{-11} to 1×10^{-6} M; in this range of concentrations acetylcholine did not alter the basal perfusion pressure. With each rate of infusion, the responses were allowed to stabilize at the new level and the mean of at least the last 4 constant responses was taken. For each artery preparation, the mean responses during infusions of acetylcholine were expressed as percentages of the mean control response. The results are shown in Figure 1. The vasoconstrictor responses were significantly increased by acetylcholine in concentrations of 4×10^{-11} M ($P < 0.01$) and 1×10^{-10} M ($P < 0.05$), and were significantly decreased by concentrations of 4×10^{-8} M ($P < 0.05$) and greater ($P < 0.01$).

In similar experiments, it was found that the increases in vasoconstrictor responses produced by low concentrations of acetylcholine (4×10^{-11} and 1×10^{-10} M) were not affected by the addition of atropine (2.9×10^{-7} M) or hexamethonium (5.5×10^{-4} M). However, the decrease in vasoconstrictor responses produced by a higher concentration of acetylcholine (1×10^{-7} M), to a mean of 25% (s.e.mean = 7.6; $n = 5$) of control, was abolished by atropine (2.9×10^{-7} M), the

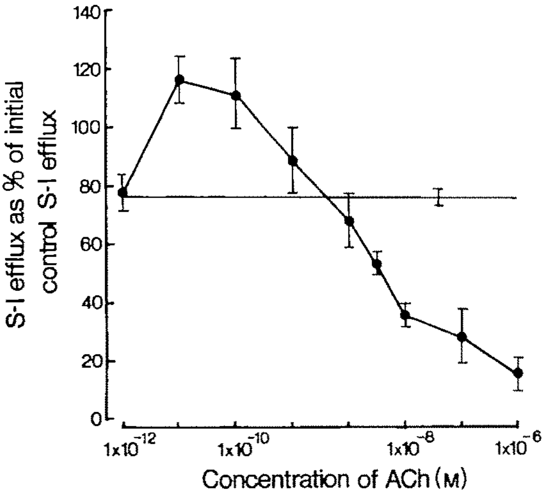

Figure 2 Effects of acetylcholine on the stimulation-induced (S-I) efflux of radioactivity from artery segments previously incubated with [^{3}H]-(−)-noradrenaline. The S-I efflux occurring in the presence of acetylcholine in the second period of stimulation was expressed as a percentage of the S-I efflux in the first period of stimulation. Symbols represent the means of 3-6 experiments and the vertical bars the standard errors of the means. The horizontal line represents the mean efflux in the second period in 7 control experiments in the absence of acetylcholine; standard error of that mean is indicated at the right hand end.

mean responses being 112% (s.e.mean = 7.6) of control. With atropine alone, the responses were 114% (s.e.mean = 9.1) of control.

Effects of acetylcholine on stimulation-induced efflux of tritium from arteries labelled with [^{3}H]-noradrenaline

Acetylcholine infusions were started 20 min before the second period of stimulation. The stimulation-induced efflux of radioactivity during the second period of stimulation was calculated as a percentage of the first stimulation-induced efflux. Thus each artery served as its own control and time-dependent changes were assessed from separate experiments in which no drugs were used. Figure 2 summarizes the effects of acetylcholine in concentrations between 1 x 10^{-12} and 1 x 10^{-6} M on the stimulation-induced efflux of radioactivity from arteries with stimulation at 5 Hz. In concentrations of 1 x 10^{-11} and 1 x 10^{-10} M the stimulation-induced effluxes were significantly enhanced ($P < 0.05$). In concentrations of 3 x 10^{-8} M and greater, acetylcholine caused significant concentration-dependent reductions in the efflux. The resting efflux of radioactivity was transiently increased by the highest concentration of acetylcholine (1 x 10^{-5} M), but after 20 min exposure, the resting efflux was not affected by acetylcholine in the range of concentrations used.

The observations made on the vasoconstrictor responses during the course of the experiments with [^{3}H]-noradrenaline were in accord with those made in the experiments with stimulation at 2 min intervals. Furthermore, the changes in efflux corresponded with the changes in vasoconstrictor response within each experiment.

The facilitatory effects of low concentrations of acetylcholine persisted after termination of the infusions; the responses and the efflux were still enhanced after 60 min of perfusion of the arteries with drug-free Krebs-Henseleit solution. On the other hand, the inhibition of responses and stimulation-induced efflux which occurred with higher concentrations of actylcholine were restored to control levels after 30 min perfusion with drug-free Krebs-Henseleit solution. In some experiments the reduction in efflux produced by acetylcholine in a high concentration was reversed to an enhancement after washout of the drug.

The simultaneous infusion of atropine (2.9 x 10^{-7} M) did not significantly alter the effect of a low concentration of acetylcholine (1 x 10^{-10} M) in enhancing the radioactive efflux and constrictor responses in 7 experiments. However, atropine prevented the reductions caused by a higher concentration of acetylcholine (1 x 10^{-5} M) in the constrictor responses and release of radioactivity: the mean efflux in the presence of acetylcholine was 16.7% (s.e.mean = 4.2; $n = 6$) of the initial control; in the presence of acetylcholine plus atropine it was 128.6% (s.e.mean = 43.8; $n = 5$); in the absence of drugs the corresponding efflux was 75.9% (s.e.mean = 2.8, $n = 7$). Infusions of atropine (2.9 x 10^{-7} M) alone did not significantly alter the stimulation-induced efflux.

In seven experiments the simultaneous infusion of hexamethonium (5.5 x 10^{-4} M) did not modify the facilitatory effect of a low concentration of acetylcholine (1 x 10^{-10} M) on the responses and accompanying efflux of radioactivity. Similarly the inhibitory effect of a higher concentration of acetylcholine (1 x 10^{-5} M) was not altered in the presence of hexamethonium. Infusions of hexamethonium (5.5 x 10^{-4} M) alone did not alter the stimulation-induced efflux or the vasoconstrictor responses.

The anticholinesterase drug, neostigmine (1 x 10^{-4} M) was without effect on the enhancement of radioactive efflux in the presence of a low concentration of acetylcholine (1×10^{-10} M). Neostigmine alone did not alter the pattern of efflux or constrictor responses in control experiments.

Discussion

The findings of Malik & Ling (1969) and Rand & Varma (1970) that low concentrations of acetylcholine increased vasoconstrictor responses to sympathetic nerve stimulation in isolated artery preparations whereas high concentrations decreased the response were attributed by these authors to increases and decreases, respectively, in the amounts of transmitter noradrenaline released since the vasoconstrictor responses to exogenous noradrenaline were not affected by the low concentrations of acetylcholine and were increased by the high concentrations. The findings have been confirmed, and it has been shown that the changes in the vasoconstrictor responses to sympathetic nerve stimulation are associated with corresponding changes in efflux of radioactivity from arteries in which transmitter stores have been labelled with [^{3}H]-noradrenaline.

In arteries previously incubated with [^{3}H]-noradrenaline, the increased stimulation-induced efflux of radioactivity in the presence of low concentrations of acetylcholine cannot be explained by block of reuptake of released noradrenaline, since acetylcholine has only a slight inhibitory effect on noradrenaline uptake in a million-fold greater concentration (Allen, Rand & Story, 1973b). The increased stimulation-induced efflux must, therefore, be attributed, to increased release of adrenergic transmitter.

The facilitatory effect of low concentrations of acetylcholine on adrenergic transmission in the rabbit ear artery was not affected by either hexamethonium or atropine. Thus the receptors involved in this action of acetylcholine are different from the nicotinic receptors on ganglion cells and the muscarinic receptors in various tissues. It is possible that the receptor site is identical with one or other of the classical nicotinic or muscarinic types, but its location is such that blocking drugs do not penetrate to it; for example, it may be intracellular and acetylcholine may have access to it through a specific transport mechanism which is not available to the blocking drug.

The inhibitory effect of high concentrations of acetylcholine on adrenergic transmission is a better known phenomenon. Brücke (1935) first demonstrated that large doses of acetylcholine can abolish the effects of sympathetic stimulation of the pilomotor muscles of the tail of the cat. These observations were confirmed by Coon & Rothman (1940) and by Burn & Rand (1960). More recently, acetylcholine has been shown to inhibit stimulation-induced release of noradrenaline, or responses to adrenergic nerve stimulation, or both, in the isolated perfused heart of the rabbit (for review, see Muscholl, 1970) and cat heart (Haeusler, Thoenen, Haefely & Huerlimann, 1968), mesenteric artery of the rat (Malik & Ling, 1969), rabbit ear artery (Rand & Varma, 1970; Hume, de la Lande & Waterson, 1972; Steinsland *et al.*, 1973), dog saphenous vein (Vanhoutte & Shepherd, 1973; Vanhoutte, Lorenz & Tyce, 1973) and cat spleen (Kirkepar, Prat, Puig & Wakade, 1972).

The inhibitory effect of acetylcholine on adrenergic transmission is antagonized by atropine and is not affected by hexamethonium, and presumably involves muscarinic receptors.

The finding that acetylcholine reduces transmitter efflux from adrenergic vasomotor nerves raises the possibility that part of the vasodilator action of acetylcholine may be attributed to this effect, but the extent to which acetylcholine-induced vasodilatation is due to this action, rather than to an action on muscarinic receptors in vascular smooth muscle, can only be resolved by further experiments directed at this question.

This work was supported by grants from the National Heart Foundation and the National Health and Medical Research Council in Australia.

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(Received September 3, 1974.
Revised October 25, 1974.)

Br. J. Pharmac. (1975), 54, 55-60

THE EFFECTS OF CYCLIC ADENOSINE 3′,5′-MONOPHOSPHATE AND GUANOSINE 3′,5′-MONOPHOSPHATE AND THEOPHYLLINE ON RENIN SECRETION IN THE ISOLATED PERFUSED KIDNEY OF THE RAT

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1 The influence of cyclic adenosine 3′,5′-monophosphate (cyclic AMP), cyclic guanosine 3′,5′-monophosphate (cyclic GMP) and theophylline on renin secretion was examined in the isolated kidney of the rat perfused with Krebs dextran solution.

2 Neither cyclic AMP (10^{-6} to 10^{-4} M) nor dibutyryl cyclic AMP (10^{-5} M) produced an increase in renin secretion.

3 Cyclic GMP and 8 Br-cyclic GMP caused a small rise in renin secretion in some experiments but this effect was independent of the dose and its physiological significance is uncertain.

4 Theophylline (10^{-6} to 10^{-4} M) caused a significant elevation in renin secretion which was not blocked by (+)-propranolol. Theophylline with cyclic AMP or cyclic GMP did not produce an amplified effect.

5 Despite previous suggestions that cyclic AMP stimulated renin secretion, this could not be confirmed in the present preparation. Since there is no evidence that cyclic AMP or cyclic GMP (or their derivatives, dibutyryl cyclic AMP and 8 Br-cyclic GMP) enter the cells, it will be necessary to study their activity in isolated juxtaglomerular cells to define a possible rôle.

Introduction

There is now much evidence that renin secretion is markedly influenced by the sympathetic nervous system and catecholamines acting on renal β-adrenoceptors (Vander, 1965; Wathen, Kingsbury, Stouder, Schneider & Rostorfer, 1965; Assaykeen, Clayton, Goldfien & Ganong, 1970; Ueda, Yasuda, Takabatake, Iizuka, Iizuka, Ihori & Sakamoto, 1970; Johnson, Davis & Witty, 1971; Winer, Chokshi & Walkenhorst, 1971; Ganong, 1972; Tanigawa, Allison & Assaykeen, 1972; Davis, 1973; Vandongen, Peart & Boyd, 1973). It is believed that the effect of many hormones on their target cells is mediated by stimulation of adenyl-cyclase leading to increase in 3′,5′ cyclic adenosine monophosphoric acid (cyclic AMP), regarded as the 'second messenger' (Robison, Butcher & Sutherland, 1971; Butcher, Robison & Sutherland, 1972). The original observation was of course that adrenaline worked in this way (Sutherland & Robison, 1966). Isoproterenol, a β-receptor stimulator, is a very effective stimulus to renin release in the isolated perfused kidney (Vandongen *et al.*, 1973) and also produces a considerable elevation of the cyclic AMP content of the kidney cortex in experimental animals (Beck, Reed & Murdaugh, 1972). Glucagon has been shown to stimulate the adenyl-cyclase/cyclic AMP system in different organs (Murad & Vaughan, 1969) and also increases renin secretion from the isolated perfused kidney (Vandongen *et al.*, 1973). This effect was not prevented by the β-adrenoceptor blocking agent, propranolol. It has been postulated that theophylline, which in isolated systems inhibits the phosphodiesterase enzyme that removes cyclic AMP, may exert its effect on whole cells by allowing accumulation of cyclic AMP (Butcher & Sutherland, 1962). This might also apply to cyclic guanosine 3′,5′-monophosphate (cyclic GMP) (Ishikawa, Ishikawa, Davis & Sutherland, 1969). Plasma renin activity was increased by theophylline in animals and in man (Winer, Chokshi, Yoon & Freedman, 1969; Reid, Stockigt, Goldfien & Ganong, 1972; Johns & Singer, 1973). It has previously been claimed that renin secretion was increased by cyclic AMP *in*

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vitro and *in vivo* (Michelakis, Caudle & Liddle, 1969; Winer *et al*, 1971, Yamamoto, Okahara, Abe, Ueda, Kishimoto & Morimoto, 1973), but this was not confirmed (Tagawa & Vander, 1970). Cyclic GMP has been viewed for a long time as a compound in search of a function (Ashman, Lipton, Melicow & Price, 1963). The kidney is rich in this substance and its physiological role is unknown (Ishikawa *et al.*, 1969) although in some secretory cells it has been suggested that there may be a common pathway, guanosine cyclase/cyclic GMP, which opposes the action of the adenylcyclase/cyclic AMP pathway (Goldberg, Haddox, Hartle & Hadden, 1973; Kolata, 1973). It therefore seemed important to determine whether cyclic AMP, cyclic GMP and theophylline had an effect on renin secretion and might indicate a final common pathway for stimulation of the juxtaglomerular cells.

Methods

Kidney perfusion

Male rats (300 g to 350 g) maintained on a regular diet were anaesthetized with sodium pentobarbitone (0.1 mg/g) intraperiteoneally and given 100 units heparin intravenously. The left kidney was isolated and perfused as described previously (Vandongen *et al.*, 1973). The perfusion fluid was Krebs-Ringer dextran saline equilibrated with 95% O_2 and 5% CO_2 at 37°C and was delivered as pulsatile flow at a constant rate (usually 8 ml/min) by roller pump. Perfusion pressure was measured by transducer and Devices M2 recorder. Experiments were begun when the perfusion pressure had stabilized at 55-80 mmHg, 6-8 min after the start. A control collection was then made during 1 min, when drug administration was started, and further similar samples were then collected every 4 minutes. The drugs were dissolved in perfusion fluid and infused separately into the arterial line through a needle in the tubing at 0.04 ml/minute. Total duration of perfusion did not exceed 30 minutes. The drug concentrations achieved in the perfusate were calculated from:

$$\frac{\text{Drug, mol/min}}{\text{Rate of flow, ml/min}}$$

Renin assay

One ml from each perfusate sample (8 ml) was dialysed for 24 h (Visking tubing) at 4°C against 0.16 M phosphate buffer (pH 4.5) containing disodium edetate, following the method for plasma renin activity (Skinner, 1967) Further dialysis against phosphate buffer (pH 7.5) for 24 h was carried out. Both buffers contained gentamycin (10 μg/ml). The samples were then incubated at 37°C with plasma from rats nephrectomized 24 h beforehand (1 ml perfusate with 0.4 ml plasma), which had been treated by similar dialysis procedures to destroy angiotensinase activity. The angiotensin I produced on incubation was measured by radioimmunoassay (Boyd, Adamson, Fitz & Peart, 1969) and the renin concentration was expressed as nanogram equivalent of 5-isoleucine-angiotensin I generated per ml of perfusate per hour of incubation. Since the peak renin concentration was reached in all experiments by 24 min from the start, results were expressed as the ratio of the renin concentration at 24 min to the renin concentration at zero time. Since flow rate was very nearly constant, concentration was used rather than secretion rate. All values given are means ± s.e. and significance was measured by Student's paired *t* test. Comparisons were always made between a control group of perfusions (*n* = 13) and the treated group.

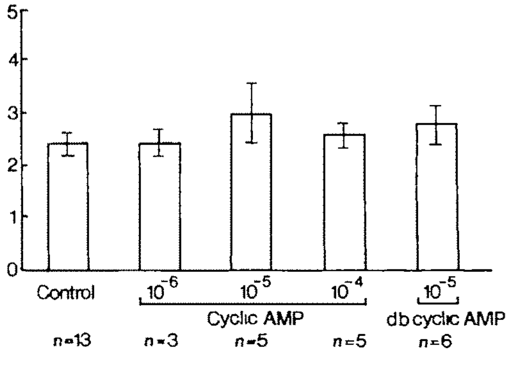

Figure 1 The effect of cyclic AMP and dibutyryl cyclic AMP (db cyclic AMP) on renin secretion in isolated perfused kidney of the rat. Molar concentrations of the drugs are expressed on the abscissae and the change of renin concentration is expressed on the ordinates as the ratio of the value at 24 min over that immediately before the start of drug administration (0 minute). Vertical bars show s.e. mean.

Materials

Adenosine 3′,5′ cyclic monophosphoric acid (cyclic AMP), N_6O_2-dibutyryl-adenosine 3′,5′ cyclic monophosphoric acid Na salt, guanosine 3′,5′ cyclic monophosphoric acid Na salt, 8 Br-guanosine 3′,5′ cyclic monophosphoric acid Na salt, theophylline (1,dimethylxanthine) (Sigma Chemical Co.) and propranolol (ICI) were used.

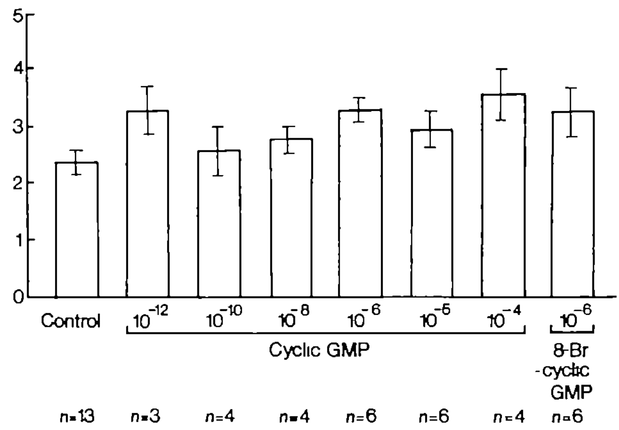

Figure 2 The effect of cyclic GMP and 8 Br-cyclic GMP on renin secretion in isolated perfused kidney of the rat. Molar concentrations of the drugs are expressed on the abscissae and the change of renin concentration is expressed on the ordinates as the ratio of the value at 24 min over that immediately before the start of drug administration (0 minute). Vertical bars show s.e. mean.

Results

Effect of cyclic AMP and dibutyryl cyclic AMP

Neither of these two substances produced an effect on renin secretion (Figure 1) and there was no alteration in perfusion pressure or flow.

Effect of cyclic GMP and 8 Br-cyclic GMP

An increase significant at $P < 0.05$ level was seen with cyclic GMP in concentrations of 10^{-4}, 10^{-6} and 10^{-12} M but with concentrations of 10^{-5}, 10^{-8} and 10^{-10} M, no significant effect was observed ($P > 0.1$) (Figure 2). Since some of the numbers of animals in the individual groups are small ($n = 3$), this might account for the variability. When 8 Br-cyclic GMP (10^{-6} M) was used, the effect was similar to that of cyclic GMP itself (10^{-6} M) (Figure 2). There was no change in perfusion pressure or flow in any experiment.

Effect of theophylline

While infusion of theophylline (10^{-7} M) did not produce a significant increase in renin secretion ($P > 0.01$), there was a significant increase of renin when the theophylline concentration was increased to 10^{-6} and 10^{-4} M ($P < 0.01$) (Figure 3). Propranolol (3.4×10^{-7} M), which was the concentration previously found to block renin release due to isoprenaline (Vandongen *et al.*, 1973), did not abolish the stimulating effect of theophylline (10^{-6} M), although the increase in renin secretion was smaller than with theophylline alone (difference not significant, $P > 0.05$). Propranolol alone was without effect (Figure 4). There was no effect on perfusion pressure or flow with theophylline or propranolol.

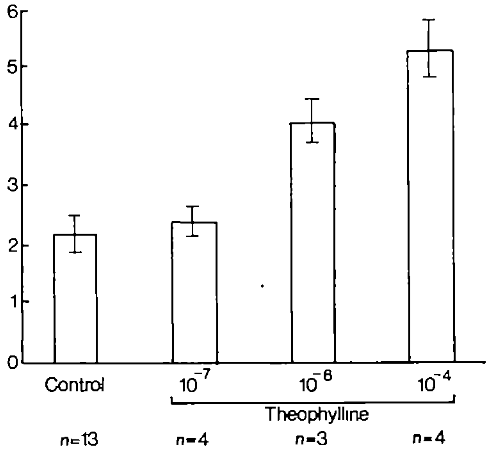

Figure 3 The effect of theophylline on renin secretion in the isolated perfused kidney of the rat. Molar concentrations of the drug are expressed on the abscissae and the change of renin concentration is expressed on the ordinates as the ratio of the value at 24 min over that immediately before the start of drug administration (0 minute). Vertical bars show s.e. mean.

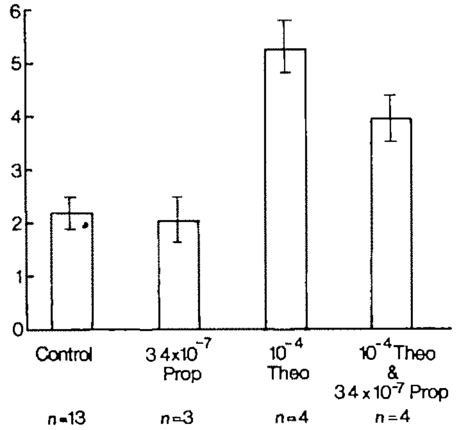

Figure 4 The effect of propranolol (Prop), theophylline (Theo) and theophylline plus propranolol on renin secretion in the isolated perfused kidney of the rat. Molar concentrations of the drugs are expressed on the abscissae and the change of renin concentration is expressed on the ordinates as the ratio of the value at 24 min over that immediately before the start of drug administration (0 minute). Vertical bars show s.e. mean.

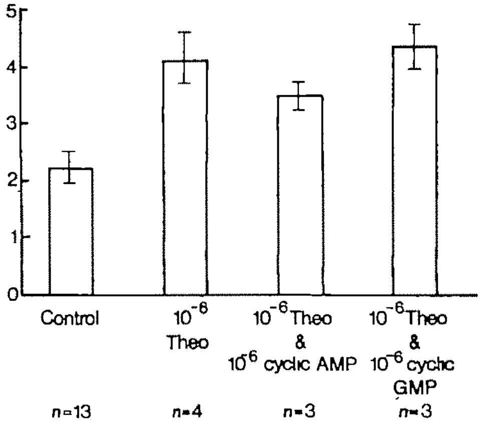

Figure 5 The effect of theophylline (Theo) plus cyclic AMP and theophylline plus cyclic GMP on renin secretion in the isolated perfused kidney of the rat. Molar concentrations of the drugs are expressed on the abscissae and the change of renin concentration is expressed on the ordinates as the ratio of the value at 24 min over that immediately before the start of drug administration (0 minute). Vertical bars show s.e. mean.

Effect of theophylline with cyclic AMP or cyclic GMP

The usual elevation of renin secretion was seen in both these studies but in neither case was the increase significantly different from that seen with theophylline alone ($P > 0.1$) (Figure 5).

Discussion

It has clearly been shown that neither cyclic AMP nor dibutyryl cyclic AMP had any effect on renin secretion. This might be because cyclic AMP could not enter the cell in sufficient quantity during the limited perfusion time (20 min) or that cyclic AMP was not on the final intracellular pathway for stimulation of renin. It has been suggested that the dibutyryl derivative passes the cell membrane more easily than cyclic AMP because it is more fat soluble and this could account for the bigger biochemical effect of this substance in some systems (Robison *et al.*, 1971; Sutherland, 1972). This idea was not confirmed in tissue culture studies (Ryan & Durich, 1972) and there is no definite evidence in its favour at the present time. It should be stated that even in experiments where cyclic AMP or dibutyryl cyclic AMP is known to stimulate biochemical processes, it is quite unknown whether this is a membrane effect or requires the entry of the substances into the cells. The present results are therefore opposed to those either in the whole animal (Winer *et al.*, 1971) or from renal cell suspensions (Michelakis *et al.*, 1969). It was of interest to study cyclic GMP not only because it occurs in the kidney but because it has been implicated in the function of some secretory cells (Eichhorn, Salzman & Silen, 1974). The very variable response at different concentrations does not carry much conviction about a real role in stimulation of renin since at best the response is not very great. The derivative 8 Br-cyclic GMP, which like dibutyryl cyclic AMP is more fat soluble, was no more active than cyclic GMP. Similar reservations apply to the evidence about its entry into cells (Estensen, Hill, Quie, Hogan & Goldberg, 1973). Theophylline has a direct effect on renin secretion without changes in perfusion pressure and flow. While it is a phosphodiesterase inhibitor, there is no evidence that this is how it releases renin and its action was not amplified by cyclic AMP or cyclic GMP. Theophylline can cause release of dopamine β-hydroxylase and noradrenaline from sympathetic nerve endings (Wooten, Thoa, Kopin & Axelrod, 1973), but there was no increase in perfusion pressure which would have been expected with sympathetic stimulation or noradrenaline release, and further this increase in renin secretion was not abolished by propranolol. This is important since it is the β-receptors that are significant in renin release. Recently it has been found that caffeine, theophylline and theobromine can decrease calcium sequestration in the sarcoplasmic reticulum in cells and can increase the calcium influx in cardiac cells (Blinks, Olson, Jewel & Branevy, 1972). While a relation between calcium flux in juxtaglomerular cells and renin release has been suggested (Vandongen & Peart, 1974), it remains uncertain how theophylline stimulates renin release A possible rôle for cyclic AMP or cyclic GMP will require study of changes in their intracellular concentration or activity in isolated juxtaglomerular cells

I. Tenyi was the recipient of a Wellcome Trust Research Fellowship

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*(Received October 22, 1974.
Revised December 30, 1974)*

Br. J. Pharmac. (1975), 54, 61-64

EFFECTS OF ACUTE COCAINE TREATMENT ON THE TURNOVER OF 5-HYDROXYTRYPTAMINE IN THE RAT BRAIN

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1 The effects of cocaine (20 mg/kg s.c.) on 5-hydroxytryptamine (5-HT) turnover were examined in rats.

2 *In vivo* cocaine administration resulted in decreased turnover of 5-HT, as indicated by the decreased accumulation of 5-HT after pargyline administration and the decreased accumulation of 5-hydroxyindoleacetic acid (5-HIAA) following probenecid injection.

3 A time-related decrease in 5-HIAA concentrations and a small fall in 5-HT concentrations in the whole brain were observed following the acute administration of cocaine hydrochloride (20 mg/kg). Tryptophan levels were found to be slightly decreased in the brain.

4 Enhanced reactivity, but neither stereotypy nor hyperthermia, was observed following cocaine injection (20 mg/kg).

5 It is concluded that cocaine inhibits the turnover of brain 5-HT and that this action of cocaine may be responsible for the differences in a number of pharmacological effects between cocaine and amphetamine.

Introduction

Much of the recent work on the effects of cocaine on the central nervous system (CNS) has been concerned with catecholaminergic systems. Cocaine blocks the neuronal uptake of tritiated dopamine (Ross & Renyi, 1967; Snyder & Coyle, 1969) and noradrenaline (Carmichael & Israel, 1973); in field-stimulated brain slices, it increases the release of catecholamines (Farnebo & Hamberger, 1971; Starke & Montel, 1973). Evidence from behavioural studies also suggests an increased catecholamine release induced by cocaine (Scheel-Kruger, 1972). Moreover, on the basis of electroencephalographic data, Wallach & Gershon (1971) have demonstrated amphetamine-like effects mediated by catecholamines after cocaine administration. However, other work suggests that cocaine acts not only on catecholamines and that an amphetamine model may be inadequate to explain all of cocaine's actions.

Simon, Sultan, Chermat & Boissier (1972), in a behavioural study, have shown that, while cocaine has amphetamine-like activity, the cocaine-induced increased locomotor activity is neither blocked by inhibition of catecholamine synthesis with α-methyl-*p*-tyrosine (α-MPT) nor by blockade of dopamine receptors with non-depressant doses of haloperidol. In addition, Knapp & Mandell (1972) have recently shown that after acute cocaine administration particulate tryptophan hydroxylase activity is decreased and that this is attributable to blockade of the high affinity uptake of tryptophan. These findings suggest that the effects of cocaine on 5-hydroxytryptamine (5-HT) turnover be examined.

Methods

5-hydroxytryptamine and 5-hydroxyindoleacetic acid (5-HIAA)

Male Sprague-Dawley rats weighing between 200 and 250 g were injected subcutaneously (s.c.) with either cocaine hydrochloride 20 mg/kg or 0.9% w/v NaCl solution (saline). After 0, 30 and 60 min, the rats were stunned and decapitated. The brains were rapidly removed, rinsed in cold saline, weighed, and homogenized in 10 volumes of cold acidified butanol. 5-HT and 5-HIAA were assayed spectrophotofluorometrically by the method of Curzon & Green (1970). Recoveries of 5-HT and 5-HIAA were 83% and 89%, respectively. The reported values have not been corrected for recovery.

Tryptophan

Sixty minutes after saline or cocaine 20 or 40 mg/kg, rats were killed and blood and brains were removed Free plasma tryptophan was estimated by assaying for tryptophan in ultrafiltrates obtained by centrifugation of 0.5 ml plasma at 900 x *g* for 30 min in Centriflo-50 membrane cones (Amicon Corp.). Tryptophan levels in both tissues were determined by the method of Denckla & Dewey (1967).

5-hydroxytryptamine turnover

Turnover of 5-HT was studied by a modification of the method of Tozer, Neff & Brodie (1966). Fifteen minutes after cocaine (20 mg/kg) or saline pretreatment, rats were injected intraperitoneally with either pargyline 75 mg/kg or probenecid 200 mg/kg and killed at various times thereafter. The effects of cocaine on turnover were evaluated by following the accumulation of 5-HT after inhibition of monoamine oxidase (MAO) with pargyline as well as the accumulation of 5-HIAA after probenecid (200 mg/kg i.p.)-induced blockade of 5-HIAA efflux from the brain.

Results

Effect of acute cocaine treatment on 5-hydroxytryptamine and 5-hydroxyindoleacetic acid concentrations in whole brain

Cocaine (20 mg/kg) caused a small decrease in brain 5-HT concentrations at all time intervals evaluated; the concentrations differed significantly from control values only at 30 min after treatment. Sixty minutes after treatment with cocaine, there was a significant ($P < 0.01$) lowering of the brain 5-HIAA concentrations. No change in 5-HIAA was seen at 30 min (Table 1)

Table 1 Effects of cocaine on 5-hydroxyindoleacetic acid (5-HIAA) and 5-hydroxytryptamine (5-HT) concentrations in whole brain

	Time (min)	*Saline*	*Cocaine*
5-HIAA (μg/g)	0	0.30 ± 0.02	0.30 ± 0.02
	30	0.29 ± 0.01	0.28 ± 0.02
	60	0.28 ± 0.01	0.22 ± 0.01*
5-HT (μg/g)	0	0.35 ± 0.02	0.33 ± 0.01
	30	0.39 ± 0.02	0.31 ± 0.02**
	60	0.36 ± 0.03	0.30 ± 0.03

Time is expressed as min after subcutaneous injection of cocaine (20 mg/kg). Each entry in the table represents the mean concentration of 5-HIAA or 5-HT with the corresponding standard error of the mean in a group of eight rats.

* $P < 0.01$ by two-tailed *t* test; ** $P < 0.02$.

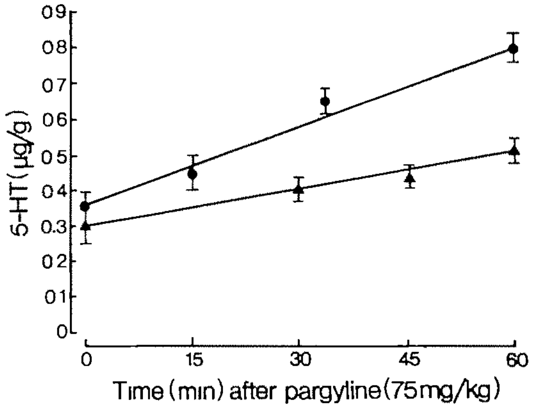

Figure 1 Effect of cocaine on the brain concentration of 5-hydroxytryptamine (5-HT) after pargyline administration Cocaine 20 mg/kg (▲) or saline (●) was injected subcutaneously 15 min before pargyline 75 mg/kg. Each point represents the mean of five determinations. Vertical bars show s.e. mean

5-hydroxytryptamine turnover

A net accumulation of 5-HT occurred over time after pargyline treatment. The rate of accumulation was decreased in the cocaine-pretreated group (Figure 1). A further indication of a reduced turnover rate in cocaine-treated rats is evident from the decreased accumulation of 5-HIAA after probenecid administration (Table 2).

Table 2 Effect of cocaine on accumulation of 5-hydroxyindoleacetic acid (5-HIAA) after probenecid administration

	Brain 5-HIAA contents (μg/g tissue)	
Time (min)	0	60
Saline	0.27 ± 0.02	0.54 ± 0.02
Cocaine	0.23 ± 0.02	0.33 ± 0.03*

Cocaine (20 mg/kg) was injected subcutaneously 15 min before probenecid (200 mg/kg). Values (μg/g) are the mean and s.e. mean of five determinations. Time (min) indicates time after probenecid administration.

* $P < 0.001$, saline vs. cocaine at 60 minutes.

Table 3 Effects of cocaine on blood and brain tryptophan concentrations

	Tryptophan concentrations		
	Plasma (μg/ml)		*Brain (μg/g)*
	Total	*Free*	
Saline	25.3 ± 0.19	0.596 ± 0.056	6.72 ± 0.11
Cocaine 20 mg/kg	26.3 ± 1.67	0.702 ± 0.066	6.28 ± 0.32
Cocaine 40 mg/kg	27.0 ± 1.41	0.470 ± 0.058	6.10 ± 0.10*

Tryptophan concentrations were determined 1 h after injection of cocaine hydrochloride. Each entry in the table represents the mean with the corresponding s.e. mean in a group of four rats.
* $P < 0.01$.

Tryptophan levels

A dose-related decrease in brain concentrations of tryptophan was observed 60 min after drug administration. However, the difference from control concentrations was significant only at the 40 mg/kg dose of cocaine. Cocaine treatment did not alter total and free plasma tryptophan concentrations (Table 3).

Behaviour

Enhanced reactivity to auditory and tactile stimulation was observed following cocaine (20 mg/kg) administration. No significant changes in rectal temperature were seen at 15, 30, or 60 min after this dose of cocaine and no evidence of motor activation or stereotyped sniffing was observed.

Discussion

The decreased rate of accumulation of 5-HT after MAO inhibition and decreased accumulation of 5-HIAA after probenecid indicate that cocaine inhibits the turnover of 5-HT. Knapp & Mandell (1972) have shown that cocaine *in vitro* decreased 5-HT synthesis by inhibition of tryptophan uptake and not by direct inhibition of tryptophan hydroxylase. However, inhibition of synthesis, especially in the presence of the blockade of 5-HT uptake known to be an effect of cocaine (Ross & Renyi, 1969), would be expected to result in a precipitous fall in brain 5-HT levels. Our finding of only a slight decrease in brain 5-HT concentrations suggests that cocaine also inhibits the release and/or metabolism of 5-HT. It is possible that cocaine stimulates 5-HT receptors directly and thus indirectly modifies release *in vivo* through a negative feedback system or that it directly inhibits 5-HT release. The effect of cocaine on 5-HT synthesis may also be partially mediated through a trans-synaptic feedback system (Andén, Corrodi & Fuxe, 1971), although tryptophan uptake appears to play a major role as shown by the *in vitro* studies (Knapp & Mandell, 1972). Our data are not sufficient to predict which, if any, of these mechanisms are involved.

The decrease in turnover as well as the postulated inhibition of release of 5-HT may be responsible for certain of the pharmacological differences between cocaine and amphetamine. Although similarities are abundant (each drug at some dose elicits stereotyped behaviour, increased locomotor activity, and similar electroencephalographic changes in mammals (Willner, Samach, Angrist, Wallach & Gershon, 1970; Wallach & Gershon, 1971; Simon *et al.*, 1972); in man, each produces euphoria, anorexia, and perceptual and affective changes at low doses and an hallucinatory psychosis that is frequently paranoid in nature at higher doses (Bejerot, 1970)), there is evidence that suggests that the action of cocaine on the catecholamines is insufficient to explain all of its effects. A study in our laboratory designed to distinguish between the effects of stimulants and the effects of psychotomimetics in chicks showed that cocaine could not be clearly classified (Wallach, Friedman & Gershon, 1972). Cocaine in low doses elicited behavioural and postural changes characteristic of amphetamine, whereas at higher doses its effects are characteristic of psychotomimetics. In addition, neither α-MPT nor haloperidol suppresses cocaine-induced hyperactivity in rats (Simon *et al.*, 1972), suggesting that the catecholamines are not responsible for these actions of the drug.

It has been proposed that certain of the psychotomimetic properties of lysergic acid diethylamide (LSD) are due to inhibition of 5-HT synthesis (Andén, Corrodi, Fuxe & Hökfelt, 1968) or to the inhibition of firing of 5-HT neurones (Aghajanian, Foote & Sheard, 1970). Snyder, Unger, Blatchley & Barfknecht (1974) have suggested that the strikingly similar clinical effects of LSD and the psychotomimetic derivatives of amphetamine or phenethylamine are due to conformational factors of the drugs that permit similar specific receptor binding of chemically distinct compounds. If these two hypotheses are valid, and if turnover of 5-HT is indeed inhibited by cocaine as our results suggest, then it is likely that this action of cocaine is responsible for the inconsistencies cited above.

This work was supported in part by NIMH Grant MH 04669

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(Received October 25, 1974.
Revised December 21, 1974.)

Br J. Pharmac. (1975), 54, 65-74

CARDIOTONIC STEROIDS: CORRELATION OF SODIUM-POTASSIUM ADENOSINE TRIPHOSPHATE INHIBITION AND ION TRANSPORT *in vitro* WITH INOTROPIC ACTIVITY AND TOXICITY IN DOGS

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1 A new series of cardiotonics based on five steroid nuclei has been evaluated for inhibition of Na^+/K^+-ATPase and Rb uptake by red blood cells, and for inotropic activity and toxicity in dogs. Structure-activity relationships are discussed.

2 The *in vitro* tests can be used satisfactorily to predict inotropic activity, but not toxicity or therapeutic ratio.

3 Although compounds with greatly improved therapeutic ratios relative to ouabain and tolusin have been obtained, they proved to be strongly emetic in the conscious dog

Introduction

The inhibition of Na^+-K^+ activated ATPase and the consequent alteration of Na^+ and K^+ fluxes by cardiac glycosides is well established, though whether this is the primary event in the inotropic effect of the glycosides is still not clear. The subject has been reviewed by Glynn (1964) and more recently by Lee & Klaus (1971). Some structure-activity relations for the inhibition of ATPase by sterols and glycosides have been demonstrated by Wilson, Sivitz & Hanna (1970) and by Shigei, Takeda, Tashima & Nakao (1971), while a large series of compounds has been investigated for ATPase inhibition, cardiotonic activity and cytotoxicity by Kupchan, Mokotoff, Sandha & Hokin (1967).

In the present work a new and more varied series of steroids has been evaluated in terms of both *in vitro* and *in vivo* activities. A primary object of the work was the improvement of the therapeutic ratio and for this a better measure of toxicity was required. The development of arrhythmias in dogs was adopted but since this test is time consuming, expensive and poor in reproducibility a correlation was sought with either ATPase inhibition or inhibition of ^{86}Rb uptake by red cells. Rubidium has been shown to be transported into red cells by the same mechanism as potassium (Bernstein & Israel, 1970) and the inhibition of this process by digoxin has been used as an assay for digoxin levels in plasma (Grahame-Smith & Everest, 1969).

Materials and Methods

Cardiotonic steroids based on the nuclei shown in Figure 1 were prepared by Mrs J. Bowler, Dr

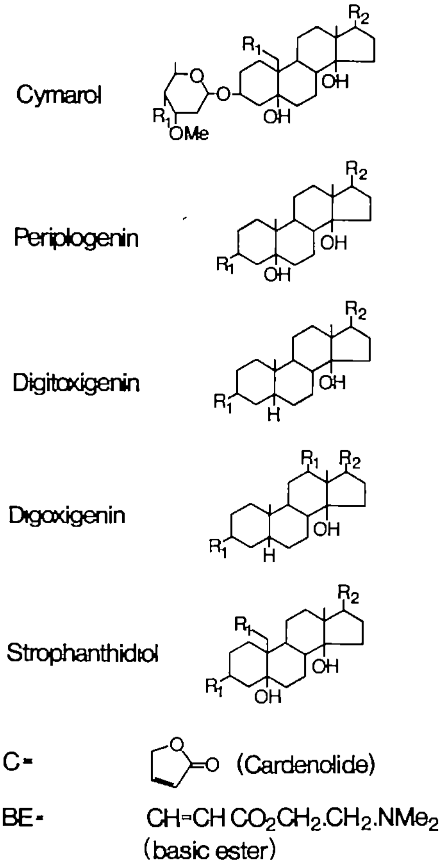


Figure 1 Structures of steroid nuclei.

R. Clarkson and Dr A. Eakin and details of their synthesis will be reported elsewhere.

Inotropic activity and arrhythmogenicity were measured in beagle dogs. The dogs were anaesthetized with pentobarbitone, 30 mg/kg, and the trachea was cannulated. The animals were artificially ventilated by intermittent positive pressure, and the thorax was opened via a right fourth intercostal space incision. A miniature pressure transducer, with frequency response uniform to 1500 Hz (Fry, 1960) was inserted into the left ventricle via the apical dimple. Left ventricular pressure (P_{LV}) so monitored was differentiated with respect to time using an active analogue differentiator circuit with output linear to 60 Hz, and to within 10% up to 90 Hz, to yield dP_{LV}/dt. Electrodes were sutured to the right atrium and intraventricular sulcus to enable atrioventricular conduction time (using a gated integrator) and ventricular rate (using a cardiotachometer) to be monitored. Lead II electrocardiogram was used to monitor arrhythmogenesis.

Aortic blood pressure was monitored via a stiff polyethylene catheter (1.5 mm bore) inserted down the right carotid artery to the level of the aortic arch. The catheter was attached to a Bell and Howell type 4-327-L221 pressure transducer driven by an SEL carrier amplifier system. All records were taken on a Mingograph 81 ink ejection oscillograph.

Increases in peak dP_{LV}/dt were taken to indicate contractility increases. Dose-response curves for peak dP_{LV}/dt and the cumulative total amount of compound given were determined in three dogs, for each individual compound and the mean amount of compound (μg/kg) necessary to cause a fixed level of peak dP_{LV}/dt increase was taken to indicate the activity of the compound. The level of increase of peak dP_{LV}/dt was set at 50% of control level since full sigmoid dose-response curve determinations are not possible with arrhythmogenic cardiotonic compounds such as those under investigation.

Peak dP_{LV}/dt changes were recorded only as percentages in all experiments with the exception of seven, in which the animals were given ouabain. In these experiments, mean control peak dP_{LV}/dt was 1777 ± 173 mmHg/second. From these experiments, peak dP_{LV}/dt, aortic blood pressure and heart rate were plotted against time. When peak dP_{LV}/dt had reached 50% over control value, changes in aortic blood pressure and heart rate were noted, and corrections were applied to peak dP_{LV}/dt using the regression relationships cited by Furnival, Linden & Snow (1970). Left ventricular end diastolic pressure changes were ignored as having little and uncertain influence on peak dP_{LV}/dt. The results of these corrections are listed in Table 1. Generally, heart rate decreased and aortic blood pressure increased as ouabain was administered at progressively higher doses. There was no significant difference between uncorrected and corrected values of peak dP_{LV}/dt Therefore, in subsequent experiments only uncorrected percentage peak dP_{LV}/dt changes were considered in order to derive compound dosages to cause 50% peak dP_{LV}/dt increases.

Toxicity of compounds was determined in animals prepared in the same way, but compounds were infused at three different rates, each in three dogs. The time of infusion for the compound given to cause regular ventricular tachycardia, with A–V dissociation, was determined, and together with the infusion rate, enabled the quantity of compound necessary to cause the specified arrhythmia to be calculated. Mean values were taken including results at all infusion rates. This assisted in overcoming some of the inherent difficulties caused by the differences in compartmental distribution rates, excretion rates, and metabolic rates possible with the different compounds. The differences between compounds in these latter respects could not be determined because of limited quantities of compound available.

With figures for both active doses and toxic doses, it was possible to determine therapeutic ratios, defined as (dose to cause arrhythmias)/(dose to cause 50% increase in peak dP_{LV}/dt).

Rate of hydroxylamine attack

The rates of hydroxylamine (0.2M) attack on cymarol analogues (5.10^{-5}M) at pH 7.3 ± 0.05 (controlled using 0.014M phosphate buffer) and 25 ± 0.1°C were obtained spectrophotometrically. The decrease in absorbance at 235 nm was measured as a function of time using a Perkin Elmer 402 u.v. recording spectrophotometer equipped with a thermostatted cell block.

The products resulting from attack were not characterized but are believed to result from addition to the double bond at C 20.

Preparation of Na^+/K^+-activated, ouabain-inhibited ATPase

Preliminary experiments showed that rat and guinea-pig heart contained only a small proportion of the Na^+/K^+-dependent enzyme as compared with dog heart and that inhibition by ouabain was poor. The majority of the rat and guinea-pig heart enzyme was Mg^{++}-dependent, Na^+/K^+-independent. It was also found that LiBr extraction by the method of Akera, Larsen & Brody (1969) gave better yields of enzyme than

Table 1 Results from seven male dogs given ouabain, with corrections to measured peak dP_{LV}/dt values according to regressions derived by Furnival *et al.* (1970).

			Control values			*Values when peak dP_{LV}/dt increase was 50%*		*Corrections to peak dP_{LV}/dt (mmHg/s)*			
Dog No.	*Weight (kg)*	*Age (Months)*	*Peak dP_{LV}/dt (mmHg/s)*	*HR (beats/min)*	*P_{AO} (mmHg)*	*HR (beats/min)*	*P_{AO} (mmHg)*	*For HR change*	*For P_{AO} change*	*Combined change*	*% Peak dP_{LV}/dt (correction at 150% control)*
1	9.5	15.5	1215	122	90	113	100	−189	+120	−69	−3.8
2	10	16.5	1975	118	118	107	144	−231	+312	+81	+2.7
3	9	16.0	2400	115	120	108	117	−147	−36	−183	−4.8
4	9	16.6	1130	125	110	125	112	0	+24	+24	+1.4
5	10	15.9	1920	130	105	119	108	−231	+36	−195	−6.7
6	9	16.2	1760	131	80	130	104	0	+288	+288	+11
7	9.5	16.7	2040	117	98	111	104	−126	+72	−54	−1.8
Means	9.4	16.2	1777	123	103	116	113				−0.28
s.e. mean	±0.2	±0.2	±173	±2.4	±5.5	±3.3	±5.6				±2.6

HR = heart rate; P_{AO} = aortic blood pressure.

NaI extraction according to Nakao, Tashima, Nagano & Nakao (1965). Dog heart extracted by the method of Akera *et al.* (1969) was, therefore, used routinely throughout this work: 20 g left ventricular muscle from a dog killed by pentobarbitone injection was minced finely with scissors and homogenized with a motor-driven Potter-Elvehjem type homogenizer (Teflon–glass) in 4 vol. of the following buffer· 0.25 M sucrose, 5 mM histidine, 5 mM disodium edetate (EDTA), 0.15% sodium deoxycholate, adjusted to pH 6.8 with tris base. The homogenate was centrifuged at 12,000 *g* for 30 min and the supernatant re-centrifuged at 100,000 *g* for 1 hour. The high speed pellet was washed by re-suspending in the same buffer and re-centrifuging at 100,000 *g* for 1 hour. This pellet was then suspended in 1 vol. 0.25 M sucrose containing 5 mM histamine and 1 mM tris EDTA and mixed with an equal vol. of LiBr (2.0 M). After stirring for 1 h at 2°C the suspension was centrifuged at 100,000 *g* for 1 h and the pellet washed by re-suspension in the same buffer and re-centrifuging under the same conditions. The final pellet was suspended in 20 ml of the sucrose/histidine/tris EDTA buffer, divided into 1 ml aliquots and stored at −20°C. Three batches prepared in this way had protein contents of 1.66, 1.07 and 1.26 mg/ml (estimated by the method of Lowry, Rosebrough, Farr & Randall, 1951). They were diluted to a standard activity before use in the assay of inhibitors.

Estimation of ATPase activity

Tris ATP (γ-^{32}P) was obtained from the Radiochemical Centre, Amersham, (to special order) as a 10 mM solution pH 7.4, 2 μCi/ml and sufficient cold 10 mM tris ATP was added before use so that 10 μl contained between 50,000 and 300,000 ct/minute The standard incubation mixture contained· 200 μl buffer (250 mM Tris, 250 mM NaCl, 75 mM KCl, 7.5 mM $MgCl_2$, pH 7.4), 100 μl enzyme suspension (25 to 36 μg protein), 100 μl H_2O (containing inhibitor if water-soluble), and 20 μl dimethyl formamide (containing inhibitor when not water-soluble). After 30 min pre-incubation at 37°C the reaction was started by addition of 100 μl ATP and stopped 20 min later by the addition of 100 μl 11% Na_3PO_4 in 7% $HClO_4$ followed by 500 μl of a suspension of charcoal in water (Norit SX 11, 60 mg/ml). After mixing rapidly in a Vortex mixer, the suspension was centrifuged in a high speed microcentrifuge and 100 μl of the supernatant added to 20 ml water for counting by Cerenkov radiation at an average efficiency of 37%. Blanks were run by adding perchlorate to the enzyme before ATP, and the Na^+/K^+ independent enzyme was estimated by omitting Na^+ and K^+ from the incubation buffer. The total counts in the incubation were also monitored by adding water instead of charcoal suspension. Per cent inhibition was calculated as·

$$100\left(1-\frac{\text{ct/min (inhibited)} - \text{ct/min (no } Na^+/K^+)}{\text{ct/min (control)} \quad - \text{ct/min (no } Na^+/K^+)}\right)$$

Uptake of Rb by red cells

$^{86}RbCl$ was received from the Radiochemical Centre, Amersham, as an aqueous solution containing 1.08 mg/ml and 5.09 mCi/ml; 10 μl of this was diluted to 1.5 ml in Krebs-Ringer-phosphate (Umbreit, Burris & Stauffer, 1957) containing 1 mg glucose/ml and 0.3 mg cold RbCl/ml, making a stock solution of 2.2 mM and 33 μCi/ml (initially) which was stored frozen at −20° C.

Whole human blood was collected in heparinized tubes and the red cells separated by centrifugation and washed twice with Krebs-Ringer-phosphate containing 1 mg glucose/ml. In the standard incubation procedure 0.2 ml red cells were diluted with 0.2 ml Krebs-Ringer-phosphate in a plastic centrifuge tube and pre-incubated with the inhibitor in 10 μl dimethyl formamide (DMF) for 45 min at 37° C; 50 μl stock RbCl solution was then added and the incubation continued for 2 h at 37° C. Ice cold 0.9% w/v NaCl solution (saline) was then added (1 ml) and the cells collected by centrifugation. After discarding the supernatant, the cells were washed three times by gentle re-suspension in 1 ml cold saline and centrifugation. The tube containing the final pellet of cells was drained and placed in a scintillation vial for counting in a scintillation counter using ^{32}P settings. About 10^5 ct/min were obtained when the ^{86}Rb was fresh and no inhibitor was present. Complete inhibition of uptake was obtained with 10^{-4}M ouabain and this was routinely used to obtain blank values (about 12,000 ct/min). Standards of 5×10^{-9}M ouabain (giving 50% inhibition) were included with each batch of compounds assayed. Per cent inhibition was calculated as·

$$100\left(1-\frac{\text{ct/min (inhibited)} - \text{blank)}}{\text{ct/min (control)} \quad - \text{blank)}}\right)$$

Fifty percent inhibition values for both ATPase and Rb uptake were obtained from plots of % inhibition vs. log concentration.

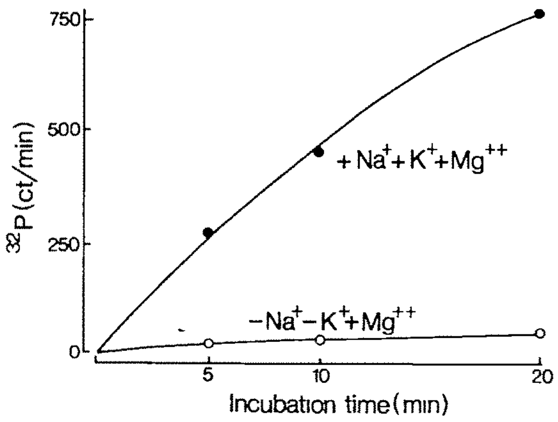


Figure 2 Dog heart ATPase. Time course of Na^+/K^+ activation.

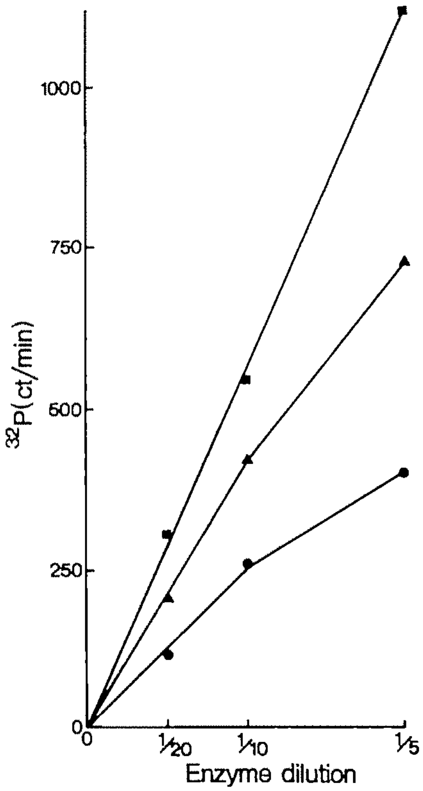


Figure 3 Dog heart Na^+/K^+ ATPase. Effects of substrate concentration and enzyme dilution: ATP 0.1 mM (●), 0.2 mM (▲), 0.4 mM (■).

Results

The ATPase prepared from dog heart by the above method has a very low content of Mg^{++} dependent

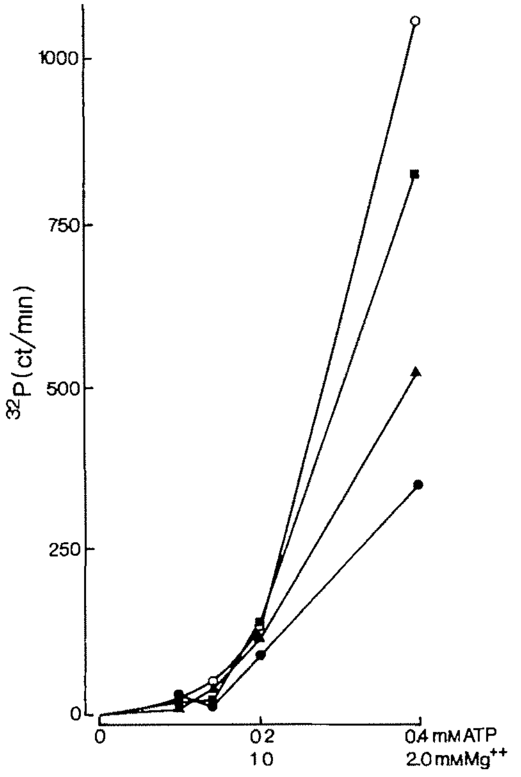


Figure 4 Dog heart Na^+/K^+ ATPase. Inhibition by ouabain at various ATP and Mg^{++} concentrations. Ouabain 10^{-6} M (●), 5×10^{-7} M (▲), 2×10^{-7} M (■), no ouabain (○).

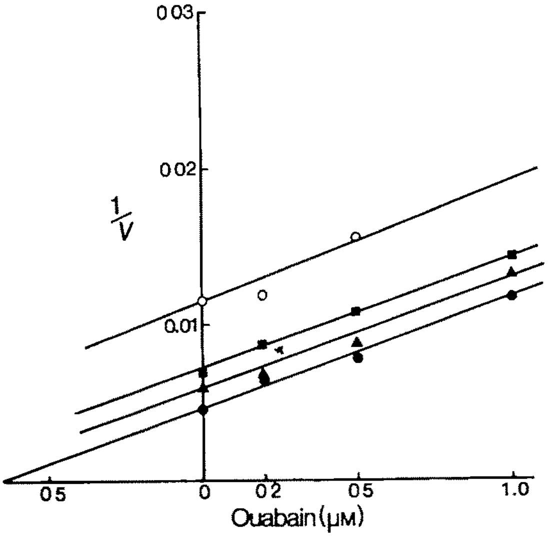


Figure 5 Dog heart Na^+/K^+ ATPase: Dixon plot for ouabain inhibition. ATP 0.4 mM (●), 0.2 mM (▲), 0.14 mM (■) and 0.1 mM (○).

enzyme and is considerably stimulated by Na^+ and K^+ (Figure 2). Its activity varies regularly with dilution and with ATP concentration up to at least 1 mM (at constant Mg^{++} concentration, Figure 3). Above 2 mM ATP there is evidence of substrate inhibition. The Na^+–K^+-dependent enzyme still requires Mg^{++} for full activity, since the substrate is believed to be an Mg^{++}–ATP complex (Hexum, Samson & Himes (1970)) and Figure 4 shows that activity below 1 mM Mg^{++}/0.2 mM ATP is so low that it is not possible to measure inhibition by ouabain accurately; 2.9 mM Mg and 1.9 mM ATP were adopted as standard for the assay of inhibitors.

The activity of the dog heart enzyme is markedly affected by an increase in temperature from 30° to 37°C although a corresponding preparation from guinea-pig heart was not. All incubations were therefore run at 37°C. A pre-incubation time of 30 min with the inhibitor before addition of ATP was employed as early experiments with guinea-pig and rat enzyme suggested that inhibition was lower without it. In fact later work with dog enzyme showed that pre-incubation made little difference. A Dixon plot (Figure 5, $1/v$ against inhibitor concentration) for ouabain inhibition gives a series of parallel lines for various ATP concentrations indicating uncompetitive (coupling) inhibition i.e. inhibition when the enzyme-inhibitor (EI) complex is not formed but the inhibitor combines only with the enzyme-substrate (ES) complex (Webb, 1963).

Inhibition of Rb uptake by ouabain is illustrated in Figure 6. Inhibition is complete at 10^{-7} M and the 50% value is 2.8×10^{-9} M. Some stimulation is apparent at concentrations of 10^{-10} M ouabain and below. A similar stimulation of chicken kidney ATPase by low concentrations of ouabain has been observed and correlated with increased ion transport in the chicken kidney tubule (Palmer & Nechay, 1964; Palmer, Lassiter & Melvin, 1966). These authors have explained the biphasic effect of ouabain in terms of two receptors on the ATPase. We have not found stimulation of our ATPase at concentrations down to 10^{-9} M ouabain.

The compounds tested were derived from the

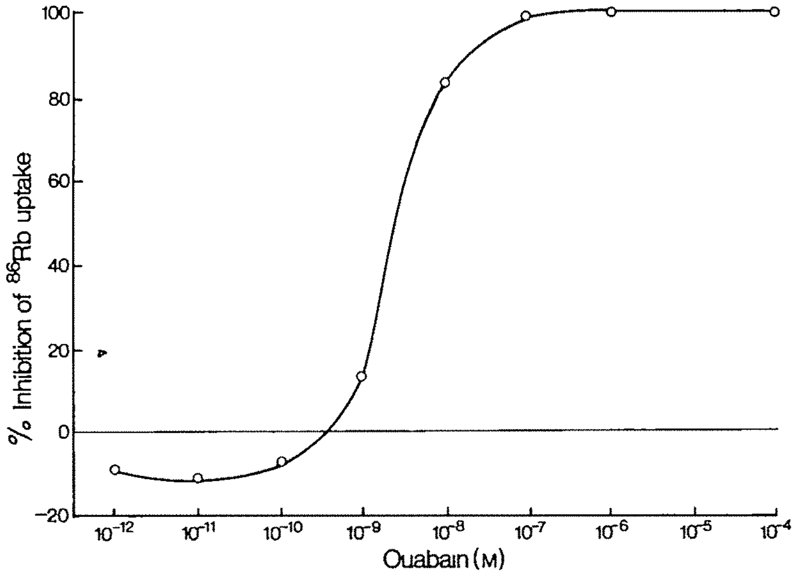


Figure 6 Inhibition of ^{86}Rb uptake into red blood cells by ouabain (for conditions see text)

five steroid nuclei shown in Figure 1. The results for ATPase inhibition, inotropic activity and inhibition of Rb uptake are given in Tables 2A-E, which are arranged to illustrate certain structure activity relations.

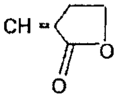
From Table 2A it can be seen that the basic esters are about as active as the corresponding cardenolides. A comparison of the activities of the compounds in Tables 2B and C shows that the activity of the cymarol analogues is markedly reduced following 4′, 19 diacylation. This is probably the effect of a bulk group on C19 since variation of the group attached to C3 has only a slight effect (compare the five periplogenin analogues in Table 2D). The high activity of the cymarol analogues (Table 2B) appears to confirm the assumption that the butenolide ring of the natural compounds may simply be replaced by an electronegatively substituted ethylene residue without loss of activity. It will be noted that the

Table 2A Comparison of basic esters and cardenolides.

Compound	*Steroid*	R_1	R_2	ATP_{50} (μM)	Rb_{50} (nM)	Δ_{50} ($\mu mol/kg$)	DA ($\mu mol/kg$)	DA/Δ_{50}
63,632	Cymarol	HO	C	.42	2.55	.055	.114	2.07
63,605	Cymarol	HO	BE	1.2	14.2	.063	.292	4.63
62,655	Cymarol	CH_3CO_2	C	47	1183			
62,838	Cymarol	CH_3CO_2	BE	21.7	391	1.3	2.83	2.18
69,654	Periplogenin	HO	C	1.2	93			
58,622	Periplogenin	HO	BE	.73	—	.095	.388	4.08
61,374	Digitoxigenin	HO	C	.32	1.07			
57,267	Digitoxigenin	HO	BE	.27	8.6	.03		
61,424	Digoxigenin	HO	C	.71	128	.21		
61,411	Digoxigenin	HO	BE	1.8	65	.056		

C = Cardenolide; BE = Basic ester.
ATP_{50} : concentration required for 50% inhibition of Na^+/K^+ ATPase.
Rb_{50} . concentration required for 50% inhibition of ^{86}Rb uptake into red blood cells.
Δ_{50} : concentration required to produce a 50% increase in dP_{LV}/dt.
DA · dose to cause arrhythmias.

Table 2B Cymarol derivatives with modified side chains (R_1 = HO).

Compound	R_2	ATP_{50} (µM)	Rb_{50} (nM)	Δ_{50} (µmol/kg)	*DA* (µmol/kg)	DA/Δ_{50}
*63,632	C	42	2.55	.055	.114	2.07
*63,605	BE	1 2	14.2	.063	.292	4 63
65,199	CH=CH CO_2H		929			
70,898	CH=CH . CN	.14	1.93	.058	.106	1.83
70,899	CH=CH . CO_2Et	.55	12.2	.16	1.055	6.59
70,900	CH=CH CO . CH_3	32	—	.18	1.39	7.72
70,901		5 5	69	.49		

* denotes compound mentioned in previous table.

Table 2C Cymarol-4′,19-diacetate derivatives with modified side chains (R_1 = CH_3CO_2).

Compound	R_2	ATP_{50} (µM)	Rb_{50} (nM)	Δ_{50} (µmol/kg)	*DA* (µmol/kg)	DA/Δ_{50}
*62,655	C	47	1183			
*62,838	BE	21.7	391	1.3	2.83	2.18
†62,966	BE	13	183	42		
65,210	CH=CH . CO_2H	NA	NA			
†63,116	CH=CH . CN	6.8	349	1.09		
62,936	CH=CH . CO_2Et	NA	NA			
65,551	CH=CH . CO . CH_3	105	3548	9.7		
63,978	CH	NA	1698			

* denotes compound mentioned in previous table; † 4′,19-dipropionates (R_1 = $C_2H_5CO_2$), NA = not active

Table 2D Basic esters (R_2 = BE).

Compound	*Steroid*	R_1	ATP_{50} (µM)	Rb_{50} (nM)	Δ_{50} (µmol/kg)	*DA* (µmol/kg)	DA/Δ_{50}
*57,267	Digitoxigenin	HO	.27	8.6	.03		
62,276	Strophanthidiol	CH_3CO_2	18	—	.94	6.78	7.21
*63,605	Cymarol	HO	1 2	14.2	.063	.292	4.63
*62,966	Cymarol	$C_2H_5CO_2$	13	183	.42		
*58,622	Periplogenin	HO	.73	—	.095	.388	4.08
63,056	Periplogenin	CH_3CO_2	.41	22	.08	.409	6.11
67,135	Periplogenin	Pr^nCO_2	.62	22.6	.09	.975	10.83
67,167	Periplogenin	$C_5H^n_{11}CO_2$	.91	2.9	.13	1.228	9.45
67,134	Periplogenin	O	.76	—	.16	1.818	11.36

Table 2E Miscellaneous compounds.

Compounds	*Steroid*	R_1	R_2	ATP_{50} (µM)	Rb_{50} (nM)	Δ_{50} (µmol/kg)	*DA* (µmol/kg)	DA/Δ_{50}
67,876	Digitoxigenin	OH	CH_2NH_2	27	241	1.9	>18.5	>9.74
67,880	Digitoxigenin	OH	$CH_2NH.CO_2-(CH_2)_2NMe_2$	3.2	—	1.4	>13.9	>9.93
61,558	Digitoxigenin	$PhCO_2$	BE	2.55	—		1.497	
Ouabain	—	—	—	.38	2.82	.028	.099	3.54
Tolusin	—	—	—	.066	.83	.075	.208	2.77

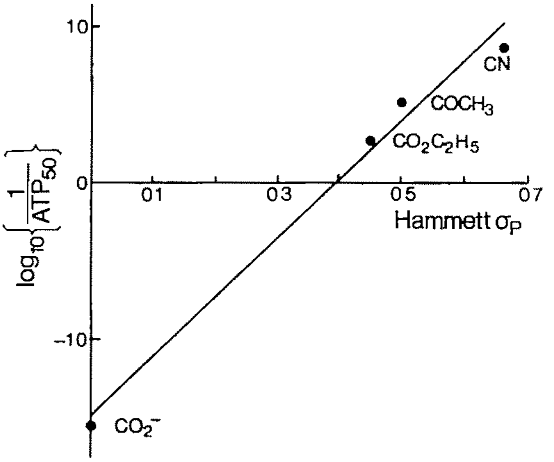


Figure 7 The relationship between ATPase inhibition data and electron withdrawal at C_{21} for cymarol analogues. Values for the Hammett σ constants are taken from Jaffe (1953).

nitrile (70,898) and the methyl ketone (70,900) are both more active than cymarol itself. In fact the ATP_{50} values fall in line with the Hammett σ values of the electronegative group (Figure 7). For these cymarol analogues a correlation was also obtained between ATPase inhibition and the rate of nucleophilic attack on the double bond (Figure 8). If one assumes that increased reactivity towards nucleophilic attack only leads to increased ATPase inhibition up to a certain maximal level then a plot of

$$\log_{10}\left\{\frac{1}{ATP_{50}}_{max} - \frac{1}{ATP_{50}}\right\}$$

versus log k_{HONH_2} is linear.

$$\left(\frac{1}{ATP_{50}}_{max} = 3.5\ \mu M^{-1}\right)$$

Most of the compounds reported by Kupchan *et al.* (1967) were reversible inhibitors and we tested the effect of washing the enzyme on the inhibition obtained with a number of our compounds. Concentrations of drug giving about 50% inhibition were used and half the enzyme samples were washed twice after incubation with the drug and before adding substrate. The other half were treated normally. The results in Table 3 show that only 63,605 (basic ester) and 70,900 (methyl ketone) are reversible. At present we have no explanation for this.

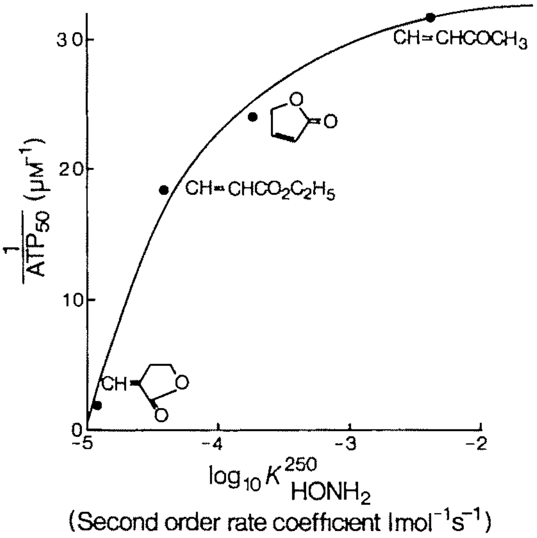


Figure 8 The relationship between ATPase inhibition data and the rate of hydroxylamine attack on the double bond of cymarol analogues. Reaction rates were measured spectrophotometrically as described in the text.

Table 3 Effect of washing on inhibition of ATPase by cardiotonic steroids.

Drug	*Conc.* *(μg/ml)*	*% inhibition of ATPase* *Control*	*Washed*
Ouabain	.73	69.3	65.9
70,898	.1	47	48
63,116	5	44.5	49
65,199	50	69.5	78
65,210	200	83.1	71 9
70,900	.5	77	0
63,605	1.0	68.1	0
62,655	50	57.6	72.5

Discussion

The desired improvement in therapeutic ratio (DA/Δ_{50}) has been achieved with a number of the compounds reported here, but on further testing in the conscious dog they proved to be highly emetic and no further development was possible. A secondary object of the work was to establish correlations between the *in vitro* and *in vivo* tests and Table 4 shows a correlation matrix from which it appears that ATPase inhibition (ATP_{50}) and rubidium uptake inhibition (Rb_{50}) correlate well with each other and with inotropic activity (Δ_{50}). The correlation of either test with toxicity

Table 4 Correlation coefficients (No. of points).

	ATP_{50}	Rb_{50}	Δ_{50}	DA	DA/Δ_{50}
ATP_{50}		.97 (21)	.98 (23)	.69 (17)	.09 (16)
Rb_{50}			99 (18)	.56 (11)	−.01 (11)
Δ_{50}				.91 (16)	.28 (16)
DA					.48 (16)
DA/Δ_{50}					

Headings as Table 1.

(DA) is less good but still significant with Rb uptake showing greater discrimination between activity and toxicity than ATPase though this may be due to the smaller number of samples for which full data were available. Neither test correlates with therapeutic ratio (DA/Δ_{50}). It is therefore not possible to use either *in vitro* test as a more precise indicator of toxicity. Haustein, Markwardt & Repke (1970) have claimed that 16-epi-gitoxin has an improved therapeutic ratio over ouabain (as measured on an *in vitro* system) which is associated with a biphasic dose-response curve for ATPase inhibition. We have obtained a sample of this compound (by courtesy of Dr Repke) and can confirm the biphasic inhibition of ATPase but in our dogs very little positive inotropic activity appeared at doses up to 100 μg/kg while arrhythmias were induced at doses as low as 5 μg/kg. All the compounds described above had linear, parallel dose-response curves (with the exception of 63,978 which had only very low activity) although many of them have greatly improved therapeutic ratios compared with ouabain. This pattern of ATPase inhibition cannot therefore be taken as indicative in general of improved therapeutic ratio.

We are indebted to Mr D Timms for the physico-chemical measurements, to Mr S.H. Ellis for statistical analysis and to Miss J M. Poole for technical assistance.

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DISSOCIATION OF THEOPHYLLINE UPTAKE AND INOTROPIC EFFECT IN MYOCARDIAL TISSUE: INFLUENCE OF TEMPERATURE, pH AND CALCIUM

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1 The myocardial uptake and the positive inotropic effect of theophylline (100 μg/ml; 0.56 mM) were studied in isolated electrically driven guinea-pig hearts perfused by the Langendorff technique under various extracellular conditions [^{3}H]-theophylline was used.

2 Variations in temperature, hydrogen ion and calcium ion concentrations of the perfusion media changed the time course and magnitude of the effect of theophylline on myocardial twitch tension but did not affect the time course and amount of theophylline uptake.

3 Under all conditions, the build-up of the positive inotropic effect of theophylline was about three times faster than the uptake of the drug into the heart.

4 Since no relationship could be found between theophylline uptake and inotropic effect, the cardiac positive inotropic response to theophylline is thought unlikely to be due mainly to an interaction of the drug with intracellular receptor sites but is assumed to occur via an effect of the drug on the sarcolemma, that is at a site which the drug reaches before it enters the intracellular space.

Introduction

Previous work of the present authors (Bellemann & Scholz, 1974) has provided evidence that theophylline enters myocardial tissue very readily. However, development of the theophylline-induced increase in myocardial twitch tension was found to proceed appreciably faster than the uptake of the drug into the heart. From these findings it was concluded that the cardiac positive inotropic effect of theophylline is unlikely to be due mainly to an action of the drug within the cell, e.g. to the release of calcium from intracellular calcium binding or storage sites.

In order to obtain further information on the relationship between theophylline uptake and the positive inotropic effect, the present work was designed to investigate whether the time course and amount of the myocardial uptake of theophylline are affected by hypothermia or by varying the extracellular hydrogen and calcium ion concentrations. Such factors are known to exert a considerable influence on cardiac mechanical performance and on drug-induced positive inotropic effects (e.g. Reiter, 1963; Blinks & Koch-Weser, 1963; Yeatman, Parmley & Sonnenblick, 1969; Scholz & de Yazikof, 1971; Reiter, 1972; Schaer, 1974).

Methods

The preparation used was the isolated guinea-pig heart perfused by the Langendorff technique. The perfusion apparatus, the methods of preparation, analytical procedure, and the experimental schedule were described by Bellemann & Scholz (1974) and only a brief account of them will be given here.

The animals (either sex, weight 380-450 g), which were kept on a standardized diet of 'Altromin' and water *ad libitum*, were injected intraperitoneally with heparin (20 mg/kg) 1 h before the experiment, killed by a sharp blow on the head and bled from the carotids. Their hearts were rapidly removed, mounted on a double-barrelled Langendorff perfusion apparatus and immediately perfused through the aorta at a constant pressure of 60 cm of water with Tyrode solution of the following composition (mM): NaCl 136.9, KCl 5.4, $MgCl_2$ 1.05, NaH_2PO_4 0.42, $NaHCO_3$ 11.9, $CaCl_2$ 0.9, glucose 5.5, (pH 7.4, 35° C); the perfusion fluid was continuously gassed with 95% O_2 and 5% CO_2. When desired, the extracellular Ca^{++}-concentration ($[Ca]_0$) was varied between 0.45 and 1.8 mM by the addition of $CaCl_2$ without allowing for the alteration of osmotic pressure. The investigations with hypothermia were performed at 25° C, $[Ca]_0$ and pH being 0.9 mM and 7.4, respectively. The perfusion medium used in the studies with different pH (temperature 35° C) was of the following composition (mM): NaCl 136.9, KCl 5.4, $MgCl_2$ 1 05, $CaCl_2$ 0.9, Tris-(hydroxymethyl)-aminomethane-HCl 5, glucose 5.5 and was bubbled constantly with 100% O_2. All perfusion fluids were filtered (glass filter, Schott 25 D 3, pore size 15-40 μm) before each experiment. The Ca^{++}-content of the perfusion medium was monitored by atomic absorption spectrometry (Perkin-Elmer, Model 403). The pH of the solutions was adjusted with a digital precision pH-meter (WTW-DIGI 610).

After dissection, all preparations were first perfused for 15-20 min with Tyrode solution under 'normal conditions' (0.9 mM Ca^{++}, pH 7.4; 35° C). The hearts were then equilibrated for 15 min with a medium of the desired experimental composition (variation of $[Ca]_0$, pH or temperature) without drug. Afterwards they were incubated for various periods (1-20 min) with the same solution containing 0.05 μCi/ml $[^3H]$-theophylline and 100 μg/ml (0.56 mM) of non-radioactive theophylline.

Constant electrical stimulation (rectangular pulses; Grass stimulator S6, frequency 3 Hz; duration 3 ms; intensity twice threshold value) during the equilibration and the experimental periods was by platinum electrodes implanted in each auricle. Mechanical and analytical measurements were performed on the same preparations. Isometric tension of the right ventricle was monitored according to Beckett (1970) with a force-displacement transducer (Grass Ft. 03) and was recorded on a Hellige Helco Scriptor recorder. Ventricular diastolic tension was adjusted to, and maintained at, 5 grams. Coronary flow was observed at 1 min intervals by measuring the effluent with a graduated cylinder.

The $[^3H]$-theophylline content of the ventricular tissue and of the incubation medium was determined as described in the previous work. Drugs used were obtained from Boehringer Sohn, Ingelheim (non-radioactive theophylline; molecular weight 180.17) and from The Radiochemical Centre, Amersham (theophylline-$[^3H]$ (G)). Formation of radioactive theophylline metabolites or tritiated water under the experimental conditions used here was not demonstrable; thus, the radioactivity measured was directly related to $[^3H]$-theophylline.

The analysis of the theophylline uptake and inotropic effect curves against time was performed according to Rescigno & Segre (1966). All data are given as means ± s.e. mean. Statistical comparisons were performed by Student's *t* test with unpaired values. A *P* value equal to or less than 0.05 was considered significant.

Results

Theophylline uptake

It is evident from Figure 1a that time course and magnitude of the theophylline uptake into myocardial tissue were neither altered by hypothermia (25° C) nor by variations of the calcium concentration and the pH of the perfusion fluid. In all cases, the uptake process proceeded very rapidly and followed the same pattern: after 3 min perfusion, the theophylline content of the hearts was already about 90% of the final 20 min level

Table 1 Amount of theophylline taken up by isolated electrically driven Langendorff-perfused hearts of guinea-pigs under different conditions

Series	*Condition* *$[Ca]_0$ mM*	 *Temperature °C*	 *pH*	*Tissue: medium ratio*
A	0.9	25	7.4	0.96 ± 0.06 (3)
	0.9	35	7.4	1.01 ± 0.02 (3)
B	0.9	35	6.6	1.01 ± 0.02 (3)
	0.9	35	7.4	1.01 (1)
	0.9	35	8.2	0.96 ± 0.04 (3)
C	0.45	35	7.4	1.03 ± 0.12 (3)
	0.9	35	7.4	1.01 ± 0.02 (3)
	1.8	35	7.4	1.02 ± 0.01 (3)

Data represent concentration of theophylline in the tissue related to concentration of theophylline in the perfusion medium (tissue: medium ratio) after 20 min perfusion with $[^3H]$-theophylline. The number of preparations is given in parentheses.

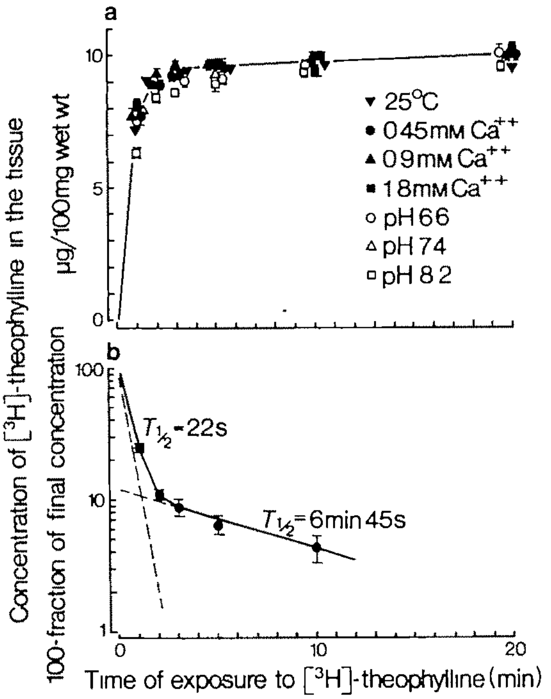

Figure 1 Isolated perfused hearts of guinea-pigs (a) Effect of hypothermia (25° C), extracellular Ca^{++} concentration (0.45-1.8 mM) and extracellular pH (6.6-8.2) on [^{3}H]-theophylline uptake $n = 3$-8 for each value. Ordinates: concentration of [^{3}H]-theophylline in myocardial tissue (µg/100 mg wet weight). Abscissae: perfusion time (min). (b) Time course of [^{3}H]-theophylline uptake. The data from Figure 1a were averaged and were replotted on semi-logarithmic coordinates according to Rescigno & Segre (1966). Each experimental point was normalized as % of, and subsequently subtracted from, the respective final value which was set at 100%. The term final value refers to the mean 20 min concentration of [^{3}H]-theophylline in the tissue as measured in each individual series of experiments. $T_{½}$ = half-time.

which, in turn, corresponded to the theophylline concentration of the perfusion medium (10 µg/0.1 ml). The latter finding can also be seen from the 20 min tissue: medium ratios listed in Table 1: they were approximately 1 under all conditions. The water content of the tissue (about 80% wet weight) also remained unaltered in all series.

More detailed information on the time course of the theophylline uptake process is given in Figure 1b. The uptake curve shown was obtained by averaging the data of all series. It is evident that theophylline entered the tissue in two phases. On the average, the first process amounted to 88% and the second one to 12% of the total uptake; the half-times of the components were 22 s and 6 min 45 s, respectively

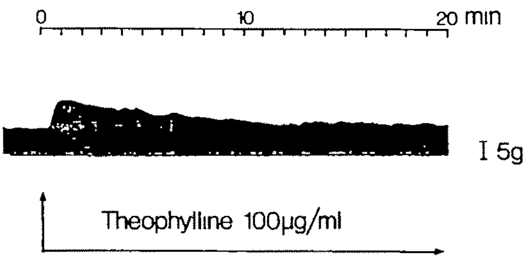

Figure 2 Isometric twitch tension in an isolated electrically stimulated (frequency 3 Hz) heart of guinea-pig perfused by the Langendorff technique before and during application of theophylline 100 µg/ml. The experiment was performed under normal conditions (temperature 35° C; pH 7.4; $[Ca]_0$ 0.9 mM)

Twitch tension and inotropic effect of theophylline

The original tracing shown in Figure 2 illustrates an experiment under 'normal conditions' (pH 7.4, 0.9 mM Ca^{++}, 35° C). The positive inotropic effect of 100 µg/ml theophylline began almost immediately after perfusing with the drug-containing medium, reached its peak within less than 1 min and then declined gradually. Under normal conditions, the time to peak effect was, on the average, 49.7 ± 2.4 s and the half-time of the build-up of the drug's positive inotropic effect was 7.6 ± 0.29 s (Table 2, series A, 2nd line). The latter value is about 3 times smaller than the half-time of even the rapid component of the theophylline uptake which was 22 s, as stated above. Thus, the former process developed appreciably more rapidly than the latter.

However, theophylline uptake and positive inotropic effect were not only dissimilar with respect to their time course. In contrast to the theophylline uptake the inotropic effect of the drug was also considerably influenced by the experimental conditions used. The build-up of the theophylline-induced increase in twitch tension was significantly slowed by acidosis (pH 6.6) and low calcium (0.45 mM) and was found to be accelerated at 1.8 mM $[Ca]_0$. This is illustrated in Table 2 whereas the respective alterations in the magnitude of the inotropic effect and in its persistence are demonstrated in Figures 3-5 (upper panels). As compared to normal conditions, the peak effect of theophylline remained unchanged

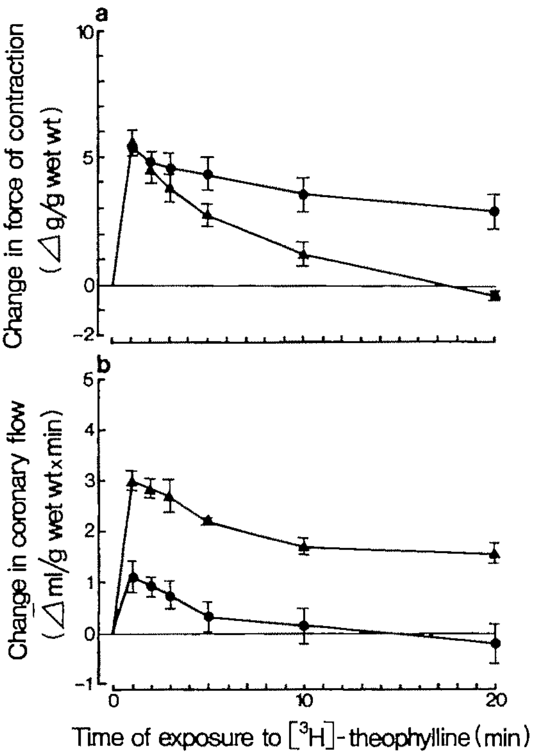

Figure 3 Effect of temperature (25° C ●, n = 3-26; 35° C: ▲, n = 3-34) upon (a) theophylline-induced changes in contractile force and (b) coronary flow of isolated perfused heart of guinea-pig. Ordinates: changes from base-line levels in force of contraction (Δ g/g wet weight) and in coronary flow (Δ ml/g wet weight x min). Abscissae· perfusion time (min). Drug addition (100 μg/ml) at zero time. Base-line values are listed in Table 3.

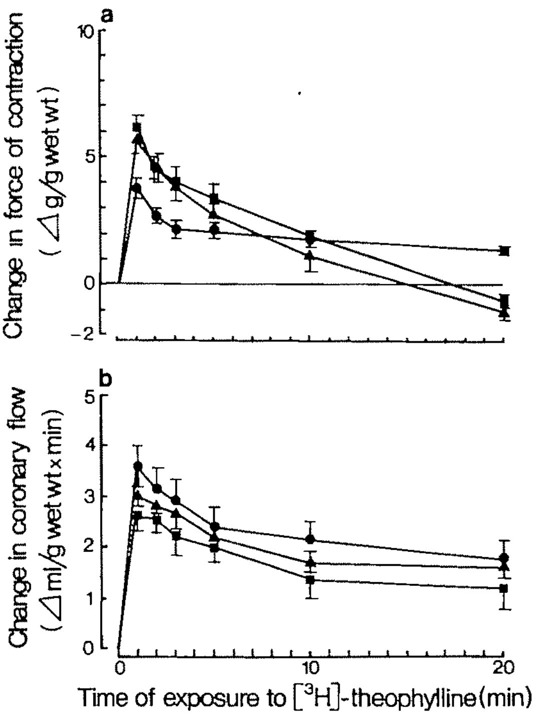

Figure 4 Effect of extracellular Ca^{++}-concentration ($[Ca]_0$ 0.45 mM ●, n = 3-18; $[Ca]_0$ 0.9 mM: ▲, n = 3-34; $[Ca]_0$ 1.8 mM. ■, n = 3-18) upon (a) theophylline-induced changes in contractile force and (b) coronary flow of isolated perfused hearts of guinea-pigs. Ordinates. changes from base-line levels in force of contraction (Δ g/g wet weight) and in coronary flow (Δ ml/g wet weight x min). Abscissae: perfusion time (min). Drug addition (100 μg/ml) at zero time. Base-line values are listed in Table 3.

Table 2 Effect of temperature (series A), pH (series B) and $[Ca]_0$ (series C) on the time to peak positive inotropic effect and on the half-time of the build-up of the positive inotropic effect in isolated electrically driven hearts of guinea-pigs treated with 100 μg/ml theophylline

Series		*Condition*			*Half-time of tension*	
	$[Ca]_0$ mM	*Temperature °C*	*pH*	*Time to peak effect (s)*	*increase (s)*	n
A	0.9	25	7.4	55.8 ± 2.6	8.4 ± 0.30	26
	0.9	35	7.4	49.7 ± 2.4	7.6 ± 0.29	34
B	0.9	35	6.6	64.6 ± 3.0^c	9.8 ± 0.55^b	19
	0.9	35	7.4	51.3 ± 3.5	7.8 ± 0.28	10
	0.9	35	8.2	55.9 ± 1.6	8.5 ± 0.60	20
C	0.45	35	7.4	59.3 ± 2.8^c	9.0 ± 0.36^a	18
	0.9	35	7.4	49.7 ± 2.4	7.6 ± 0.29	34
	1.8	35	7.4	37.3 ± 2.1^b	5.6 ± 0.26^b	18

Values represent means ± s.e. mean. n = number of preparations.

^{a,b,c} Significant difference from values measured under normal conditions (0.9 mM Ca^{++}, pH 7.4, 35°C).

^{a} $P < 0.001$; ^{b} $P < 0.01$; ^{c} $P < 0.02$.

with hypothermia (Figure 3), 1.8 mM Ca^{++} (Figure 4), and alkalosis (Figure 5) but was smaller at pH 6.6 (Figure 5) and 0.45 mM Ca^{++} (Figure 4). Finally, the decline in tension development between the peak inotropic effect and the end of theophylline perfusion was considerably slower with hypothermia (Figure 3) and in low calcium medium (Figure 4).

It is evident from Table 3 that the predrug force, i.e. the level before theophylline application, was also altered by hypothermia or variations of the extracellular calcium and hydrogen ion concentrations. These results are in accordance with previous work (e.g. Reiter, 1963; Kohlhardt, Wirth & Dudeck, 1967; Pannier & Leusen, 1968; Cingolani, Mattiazzi, Blesa & Gonzales, 1970; Scholz & de Yazikof, 1971; Reinhardt, Wagner & Schümann, 1972, Kammermeier & Rudroff, 1972; Schaer, 1974). Because of these variations in predrug force, the theophylline-induced changes in twitch tension given in Figures 3-5 are not expressed as a percentage of the base-line tension but, instead, as Δg/g wet weight, this is as absolute change from the respective control level (cf. Rall & West, 1963). The same applies to the coronary flow values described below.

Coronary flow

Under normal conditions, the predrug coronary flow amounted to 6.6 ± 0.2 ml/g wet weight x min (Table 3, series A, 2nd line). It was enhanced by hypothermia and acidosis, was diminished by alkalosis but was not altered by changes in [Ca]$_0$ (Table 3). Theophylline increased coronary flow in all series (Figures 3-5; lower panels); as compared

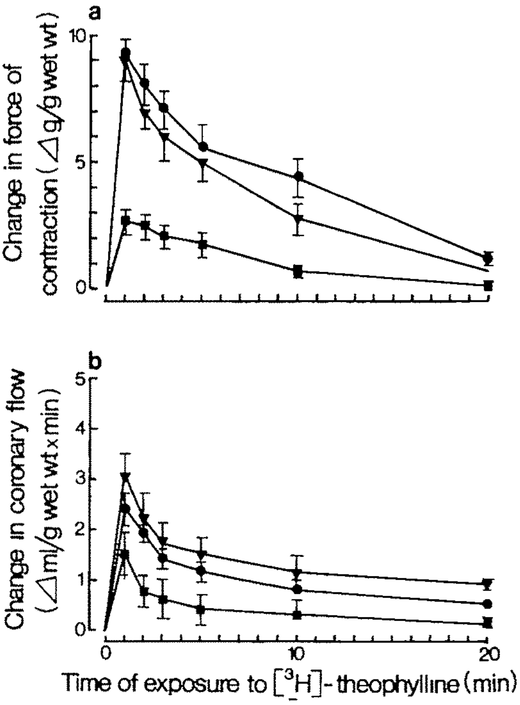

Figure 5 Effect of extracellular pH (pH 6.6 (■), n = 3-19; pH 7.4 (▼), n = 2-10; pH 8.2 (●), n = 3-20) upon (a) theophylline-induced changes in force of contraction and (b) coronary flow of isolated perfused hearts of guinea-pigs. Ordinates: changes from base-line levels in contractile force (Δ g/g wet weight) and in coronary flow (Δ ml/g wet weight x min). Abscissae: perfusion time (min). Drug addition (100 µg/ml) at zero time. Base-line values are listed in Table 3.

Table 3 Effect of temperature (series A), pH (series B) and [Ca]$_0$ (series C) on force of contraction and coronary flow of isolated electrically driven hearts of guinea-pigs as measured immediately before application of theophylline

Series		*Condition*			
	[Ca]$_0$ mM	*Temperature °C*	*pH*	*Force of contraction g/g wet weight*	*Coronary flow ml/g wet weight x min*
A	0.9	25	7.4	13.4 ± 0.8 (31)^b	8.0 ± 0.2 (31)^a
	0.9	35	7.4	10.6 ± 0.6 (34)	6.6 ± 0.2 (34)
B	0.9	35	6.6	0.9 ± 0.1 (22)^a	10.0 ± 0.8 (22)^b
	0.9	35	7.4	5.9 ± 0.6 (10)	6.5 ± 0.5 (10)
	0.9	35	8.2	12.5 ± 1.2 (20)^a	5.1 ± 0.2 (20)^b
C	0.45	35	7.4	3.8 ± 0.3 (18)^a	6.6 ± 0.3 (18)
	0.9	35	7.4	10.6 ± 0.6 (34)	6.6 ± 0.2 (34)
	1.8	35	7.4	13.0 ± 0.7 (18)^c	6.5 ± 0.3 (18)

The number of preparations is given in parentheses. Values represent means ± s.e. mean.
^{a,b,c} Significant difference from values measured under normal conditions (0.9 mM Ca^{++}, pH 7.4, 35°C).
^{a} $P < 0.001$; ^{b} $P < 0.01$; ^{c} $P < 0.02$.

to normal conditions, the effect of theophylline was diminished by hypothermia (Figure 3) and, to a smaller extent, by acidosis (Figure 5) but remained unaltered by alkalosis (Figure 5) and the different calcium concentrations studied (Figure 4).

Discussion

We have previously shown that the uptake of theophylline into the mammalian heart proceeds very rapidly and that the steady-state concentration of theophylline in the myocardial tissue corresponds to that of the perfusion fluid (Bellemann & Scholz, 1974). These findings led us to suggest that the myocardial uptake of theophylline is mainly by rapid diffusion and that the drug distributes itself in the heart muscle as freely as in the Tyrode solution. The present results are in accord with this view and allow additional conclusions. With respect to the mechanism of the uptake process, the most meaningful observations of this study are that the myocardial uptake of theophylline is not affected by hypothermia (25° C) or by varying the extracellular pH within a range of 6.6-8.2. From the former finding it can be concluded that the theophylline uptake is unlikely to be mediated by an energy-dependent process. The latter result suggests that theophylline which is a weak acid (protòn at N-7; pK_a 8.77; Merck Index, 8th ed., p. 1034) as well as a negligibly weak base (pK_b 13.72) is mainly taken up as an uncharged molecule, at least in the pH range studied. The present results thus confirm and extend our previous conclusions which can now be summarized thus: theophylline moves through the cardiac cell membrane mainly by nonspecific passive diffusion and not by an active process or by electrical forces.

The second point to be discussed is the lack of coincidence between theophylline uptake and inotropic effect. Such dissociation which is felt to be relevant with respect to the mechanism of the inotropic response to the drug applies firstly to the different time courses of both events, and secondly to their dissimilar responsiveness to variations in the experimental conditions.

In all series of experiments, the positive inotropic effect of the drug developed appreciably more rapidly than even the fast component of the theophylline uptake. This finding corroborates our previous results (Bellemann & Scholz, 1974) and suggests that the 'receptor' involved in the development of the inotropic effect is mainly located at a site which the drug reaches before it enters the intracellular space. It appears not unreasonable to assume that this site is the sarcolemma because previous electrophysiological work has provided evidence that theophylline increases the slow calcium inward current during the cardiac action potential and that this effect is closely related to the inotropic response to the drug (Scholz, 1971).

However, theophylline uptake and the inotropic effect were not only dissimilar with respect to their time course but also with respect to their different susceptibility to changes in the extracellular conditions. For instance, the rate of development and the peak magnitude of the positive inotropic effect of the drug were diminished by acidosis and by reducing the extracellular calcium concentration but the rate and amount of theophylline uptake were not affected by the same changes. On the other hand, elevation of the extracellular calcium concentration was found to accelerate the development of the inotropic response to theophylline but the theophylline uptake again remained unchanged. These dissociations further support the view that there is no direct cause and effect relationship between the uptake of theophylline into the myocardial cell and the inotropic response to the drug or, in other words, that the former event, although rapid and well pronounced, is not a prerequisite for the latter.

The effects of pH or $[Ca]_0$ changes upon the cardiac inotropic response to theophylline described here correspond to those previously observed with catecholamines (e.g. Reiter & Schöber, 1965; Schaer, 1974). The altered activity, e.g. with variations of pH, of the latter agents may be attributed to a changed sensitivity to catecholamines of transmembrane calcium fluxes (Morgenstern, Noack & Köhler, 1972), of calcium sequestration by the sarcoplasmic reticulum (Chimoskey & Gergely, 1968), of the cyclic AMP-system (Reynolds & Haugaard, 1967), or even to nonspecific drug-independent changes in myocardial contractility (Schaer, 1974). Unfortunately, similar data with theophylline are as yet not available so that the mechanism underlying the effects of variations of pH or $[Ca]_0$ upon theophylline action on cardiac mechanical behaviour remains to be elucidated. However, from the present results it can be concluded that changes in the access of theophylline to its receptor site or interference with metabolic degradation of the drug during incubation are unlikely to be significant factors.

This work was supported by the Deutsche Forschungsgemeinschaft (Scho 15/2). The expert technical assistance of Mrs Hildegard Wessling-Seufert is gratefully acknowledged.

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STUDIES ON THE POSITIVE INOTROPIC EFFECT OF PHENYLEPHRINE: A COMPARISON WITH ISOPRENALINE

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1 The effects of phenylephrine and isoprenaline on the isometric contraction of guinea-pig ventricle were compared over the whole range of their respective dose-response curves.

2 In preparations driven at 2.5 Hz the increase in contractile force induced by either isoprenaline or phenylephrine was linearly correlated to an increase in maximum velocity of force development. The relaxation time was shortened by isoprenaline but not by phenylephrine.

3 The negative inotropic effect induced by δ[N-(3,4-dimethoxyphenethyl)-*N*-methylamino]-α-(3,4,5-trimethoxyphenyl)α-isopropylvaleronitrile hydrochloride (D_{600}) was reversed by isoprenaline, but little influenced by phenylephrine.

4 The study of the interval-force relationship shows that the increase in contractile force induced by phenylephrine (3×10^{-5} M) was relatively greater at low frequencies of stimulation, and that the maximum effect was reached at the frequency of 1 Hz.

5 The positive inotropic effect of phenylephrine (10^{-4} M) was significantly higher at a frequency of 1 Hz than at 2.5 Hz; the effect of isoprenaline (3×10^{-8} M) was not significantly different at the two driving frequencies.

6 In preparations driven at 1 Hz the inotropic effect of the lower concentrations of phenylephrine was due to an increase in the time to peak tension without any change of the maximum velocity of force development; however an increase of this parameter became evident only after higher concentrations of the amine (10^{-5} M or more), associated with a progressive shortening of the time to peak.

7 A correlation between mechanical and electrophysiological effects of phenylephrine is attempted, the suggestion is advanced that the prolongation of the action potential and of the active state duration may be an important factor in the inotropic effect of phenylephrine.

Introduction

It is now widely accepted that the positive inotropic effect of phenylephrine is mediated through an activation of myocardial α-adrenoceptors (Benfey, 1973). In the rat ventricle (Wenzel & Su, 1966), rabbit atria (Benfey & Varma, 1967; Parr & Urquilla, 1972) and guinea-pig atria (Govier, 1967, 1968) the increase in contractility induced by the amine is inhibited by α-adrenoceptor blocking drugs.

The positive inotropic effect of phenylephrine is independent of effects on adenylcyclase and cyclic adenosine 3,5'-monophosphate (cyclic AMP) concentration (Benfey, 1971; Benfey & Carolin, 1971); moreover theophylline, in a concentration sufficient to inhibit phosphodiesterase activity and to potentiate the effect of isoprenaline, does not influence the inotropic action of the α-receptor stimulating amine (Hamakawa, Shimizu & Toda, 1973).

It has been demonstrated that the activation of myocardial α-adrenoceptors is associated with a prolongation of the action potential duration (Pappano, 1971; Giotti, Ledda & Mannaioni, 1973). An increase of the cardiac action potential duration mainly due to a lengthening of the plateau phase is induced by phenylephrine in a concentration-dependent manner (Ledda, Marchetti & Manni, 1971; Ledda & Marchetti, 1971). These observations led us to advance the hypothesis that the positive inotropic effect of phenylephrine could be due, at least in part, to a prolongation of the duration of the active state. Therefore in this study the effects of phenylephrine on the isometric contraction of guinea-pig

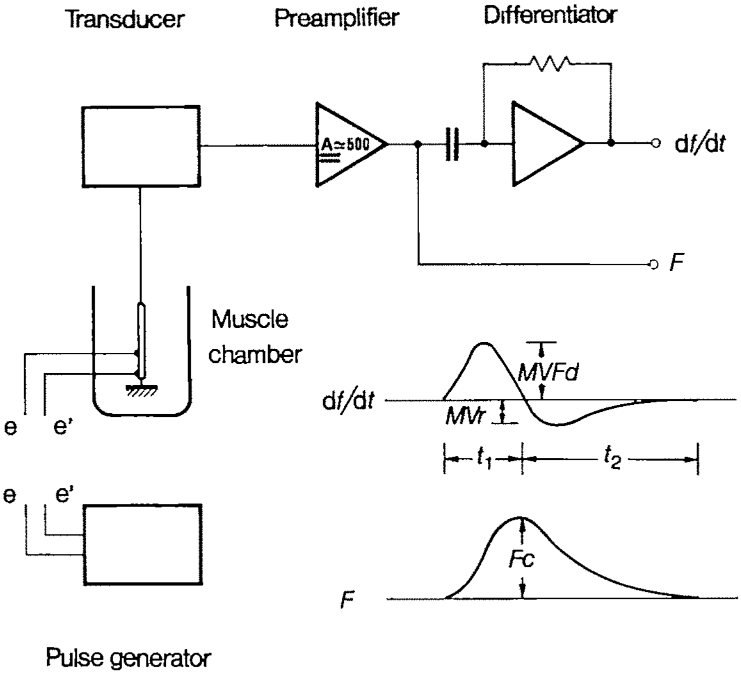

Figure 1 Block diagram of the experimental set-up, showing the isometric contraction curve, the first derivative, and the measurements taken.

ventricle were observed over the whole range of the dose-response curve. In addition the effect of the amine was compared with that of isoprenaline to obtain some information about the possible differences between the mechanical responses elicited in cardiac muscle by α- and β-adrenoceptor agonists.

Methods

Isolated ventricle strips (about 2 mm wide and 10 mm long) obtained from guinea-pig hearts were mounted vertically in a double walled chamber containing 20 ml of perfusion fluid with the following composition (mM): NaCl 115; KCl 4.7; $CaCl_2$ 3.6, $MgSO_4$ 1.2, KH_2PO_4 1.2; NaH_2CO_3 25; glucose 10. The solution was constantly gassed with a mixture of 97% O_2 and 3% CO_2, and kept at 30° C.

The preparations were stimulated electrically at constant rates (1 and 2.5 Hz) by square wave pulses delivered by a pulse generator.

The length of the muscle was adjusted by stretch to produce a resting force of 0.8 g; the resting force was then maintained constant during the whole experiment.

The isometric contraction curves were obtained by an isometric transducer and a d.c. preamplifier; the first derivative of tension was obtained by a differentiator (operational amplifier) with a linear output between 0 and 100 V/second. The two variables were displayed on a dual beam oscilloscope, and were photographed at high sweep speed (100 ms/cm) by means of a Grass C4-K camera.

The following measurements were taken (Figure 1): *Fc* (force of contraction); t_1 (time to peak tension), t_2 (relaxation time); *MVfd* (maximum velocity of force development), *MVr* (maximum velocity of relaxation); ΔFc (inotropic effect change in peak force of contraction).

The experiment started after a 30-60 min period of equilibration. Cumulative dose-response curves were obtained according to the technique described by Van Rossum (1963), with the 1/2 $\log_{10}$ procedure.

Tension-frequency curves were obtained by raising stepwise the driving frequency from 0.1 to 4.0 Hz; constant frequencies of stimulation were maintained until a steady state contractile tension was attained.

The following substances were used: (±)-isoprenaline hydrochloride (Fluka), (—)-phenylephrine hydrochloride (K and K Lab.), δ[N-(3,4-dimethoxyphenethyl)-*N*-methylamino] -α-(3,4,5-

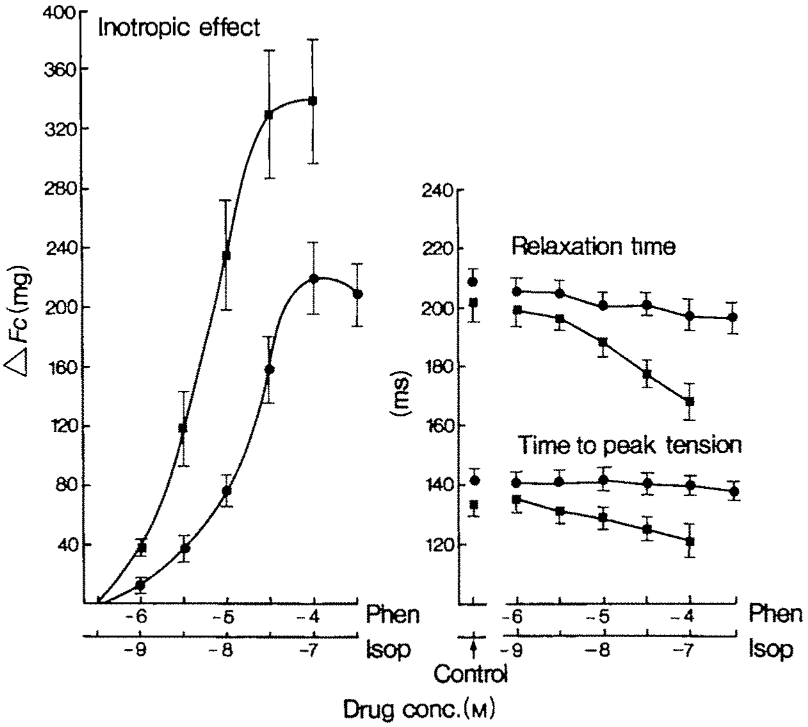

Figure 2 Inotropic effect, relaxation time and time to peak tension as influenced by phenylephrine (mean values of 12 experiments. (●)) and isoprenaline (mean values of 11 experiments: (■)) in cumulative concentrations. Vertical bars show s.e.mean. Stimulation frequency· 2.5 Hz. Temperature· 30° C.

trimethoxyphenyl)α-isopropylvaleronitrile hydrochloride (D_{600} hydrochloride, kindly supplied by Knoll).

Results

Inotropic effects in preparations driven at 2.5 Hz

Cumulative dose-inotropic response curves for isoprenaline and phenylephrine are shown in Figure 2. A positive inotropic effect was induced by isoprenaline in concentrations ranging from 10^{-9} to 10^{-7} M; the phenylephrine effect was obtained with concentrations ranging from 10^{-6} to 10^{-4} M. The endpoint of the concentration-effect curve for isoprenaline was considerably higher than that for phenylephrine: the maximum increase of contractile force (ΔFc) induced by the β-adrenoceptor agonist (10^{-7} M) was 337.1 ± 53.6 mg, while that induced by the α-adrenoceptor agonist (10^{-4} M) was 220.0 ± 23.5 (mean values of 7 and 12 experiments respectively).

Besides this quantitative difference in the inotropic effect, the two amines also had different effects upon the shape of the isometric contraction curves. Isoprenaline shortened the relaxation time (t_2) whereas this parameter was not affected by phenylephrine.

Time to peak tension (t_1) was only slightly and not significantly affected by both substances (Figure 2).

The increase in contractile force (ΔFc) induced by either isoprenaline or phenylephrine was linearly correlated to the increase in maximum velocity of force development ($MVfd$): the correlation coefficients (r) are 0.908 and 0.803, and the regression coefficients (b) are 0.020 and 0.023 respectively.

A straight correlation was also found between ΔFc and MVr (r = 0.927 and b = 0.018 for isoprenaline; r = 0.889 and b = 0.020 for phenylephrine).

Reversal of D_{600}-induced negative inotropic effect

D_{600}, a methoxyderivative of verapamil, is a negative-inotropic agent which can inhibit excitation-contraction coupling (Fleckenstein, 1971), by

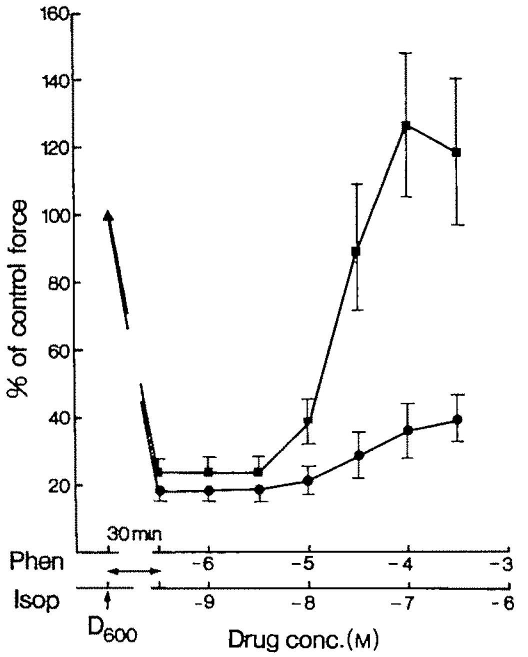

Figure 3 Reversal of the D_{600} (0.25 µg/ml)-induced negative inotropic effect by phenylephrine (mean values of 5 experiments: (●)) and isoprenaline (mean values of 5 experiments: (■)) in cumulative concentrations. Vertical bars show s.e.mean. Stimulation frequency· 1.5 Hz. Temperature 30° C.

selectively blocking the calcium transmembrane influx into the excited myocardial fibres (Kohlhardt, Bauer, Krause & Fleckenstein, 1972). The contractile tension of preparations stimulated at 2.5 Hz was reduced by about 80% after treatment for 30 min with 0.25 µg/ml of the drug.

Isoprenaline added in a cumulative way reversed the D_{600}-induced negative inotropic effect: the contractile tension, which decreased to 23.3 ± 4.3% of control values, returned to 126.7 ± 21.8% after isoprenaline 10^{-7} M.

Phenylephrine, even at the highest concentration (3×10^{-4} M), scarcely influenced the effect of the negative inotropic agent: the contractile tension, decreased to 18.08 ± 4.3% by D_{600}, was increased only to 40.2 ± 7.4% after phenylephrine 3×10^{-4} M (Figure 3).

Frequency-dependence of inotropic effects

Tension frequency curves obtained from 6 preparations before and 5 min after treatment with phenylephrine at a concentration able to induce a submaximal inotropic effect (3×10^{-5} M) showed an upward shift induced by the amine (Figure 4). Moreover the interval-force relationship was altered in some respects: the increase in contractile force induced by phenylephrine was relatively higher at low frequencies of stimulation, and a maximum was reached at the frequency of 1 Hz, as the interval between beats was further shortened, the decrease in contractility was earlier in the preparation treated with phenylephrine than in controls. The positive inotropic effect of phenylephrine is either quantitatively or qualitatively dependent on stimulation rate.

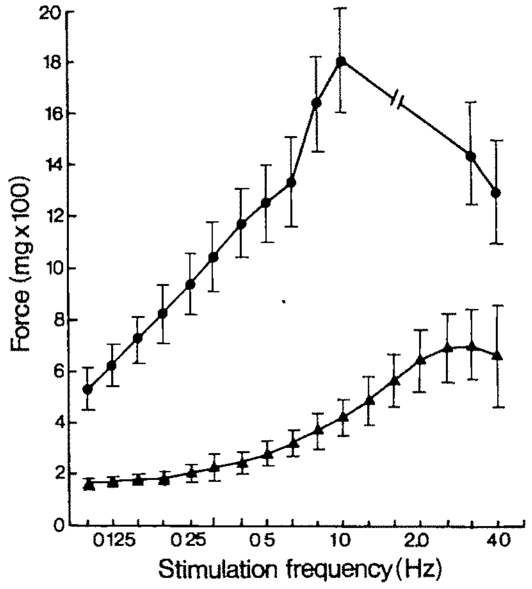

Figure 4 Tension-frequency curves obtained in 6 preparations before (▲) and 5 min after phenylephrine 3×10^{-5} M (●); mean values are given. Vertical bars show s.e.mean. Temperature 30° C.

Table 1 shows that the net increase in contractile force induced by phenylephrine 10^{-4} M was significantly higher ($P < 0.01$) in preparations driven at 1 Hz than in those driven at 2.5 Hz.

A comparison between the maximum inotropic effect of isoprenaline and phenylephrine at the lower frequency of stimulation was made impossible by the frequent development of spontaneous high frequency arrhythmias induced by isoprenaline concentrations above 3×10^{-8} M in preparations driven at 1 Hz. For this reason a concentration of 3×10^{-8} M isoprenaline was chosen for the comparison between the effects of the two amines at the two frequencies of stimulation. As shown in Table 1, the net increase of contractile tension induced by phenylephrine 10^{-4} M was significantly higher ($P < 0.01$) than that induced by isoprenaline 3×10^{-8} M at 1 Hz

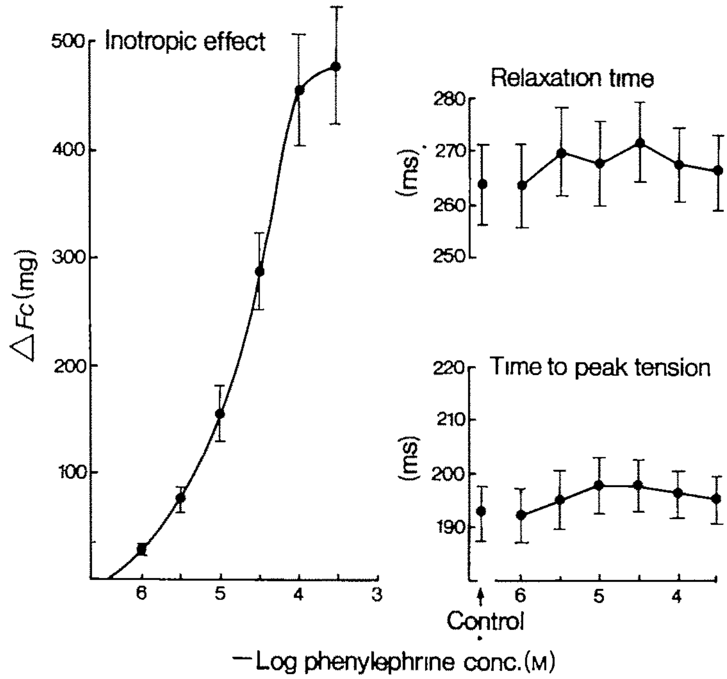

Figure 5 Inotropic effect, relaxation time and time to peak tension as influenced by phenylephrine in cumulative concentrations. Mean values of 11 experiments; vertical bars show s.e.mean. Stimulation frequency. 1 Hz. Temperature 30° C.

whereas the reverse is true at 2.5 Hz ($P = 0.05$). The effects of isoprenaline 3 x 10^{-8} M were not significantly different at the two driving frequencies.

With regard to qualitative differences Figure 5 shows that the inotropic effect of phenylephrine at 1 Hz was associated with a slight increase of both the time to peak tension and the time of relaxation, whereas no variations of the two parameters were detectable at 2.5 Hz (for comparison see Figure 2).

Although these changes in isometric contraction parameters do not reach the level of statistical significance, because of considerable variation among preparations, individual tracings often showed a distinct difference between the effects of

Table 1 Differences between the inotropic effects of isoprenaline (3 x 10^{-8} M) and phenylephrine (10^{-4} M) in preparations driven either at 1 Hz or at 2.5 Hz

Drug (molar concentration)	*Net increase of contractile tension (ΔFc) (mg)*	
	1 Hz	*2.5 Hz*
Isoprenaline (3 x 10^{-8} M)	A_1 285.0 ± 43.2 (7)	A_2 329.0 ± 41.7 (11)
Phenylephrine (10^{-4} M)	B_1 460.4 ± 54.1 (11)	B_2 220.0 ± 23.5 (12)

Statistical significance of differences*:
A_1-A_2 = NS
B_1-B_2 = $P < 0.01$
A_1-B_1 = $P < 0.01$
A_2-B_2 = $P = 0.05$

Number of experiments in parentheses.

* Statistical analysis was performed according to the variance analysis of a factorial experiment (m x n) followed by *t* test of non orthogonal comparisons.

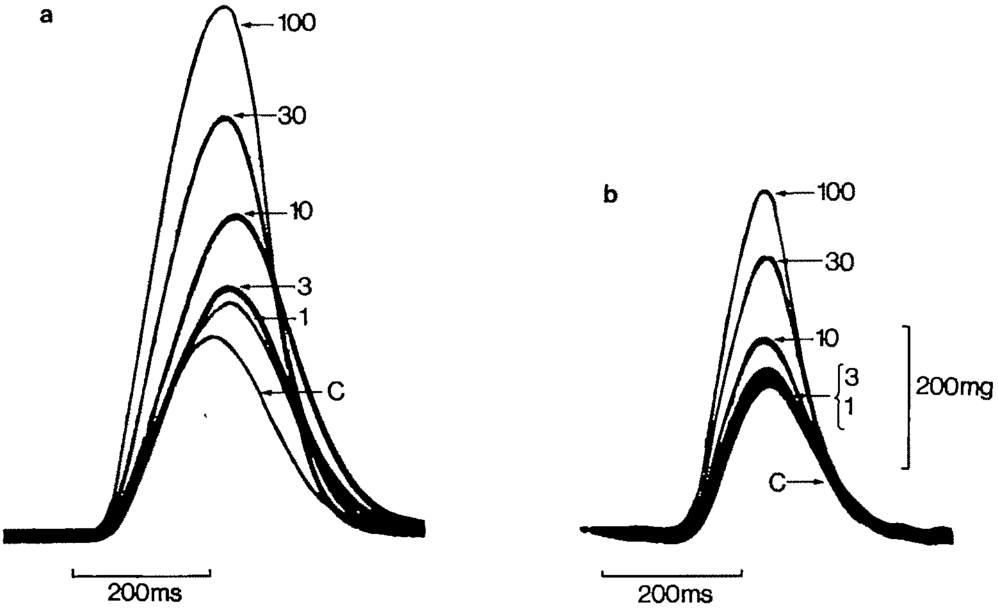

Figure 6 Superimposed concentration-inotropic effect curves obtained in the same preparation treated with phenylephrine and driven either (a) at 1 Hz or (b) at 2.5 Hz. Numbers indicate drug concentration (x10^{-6} M); C = control. Temperature: 30° C.

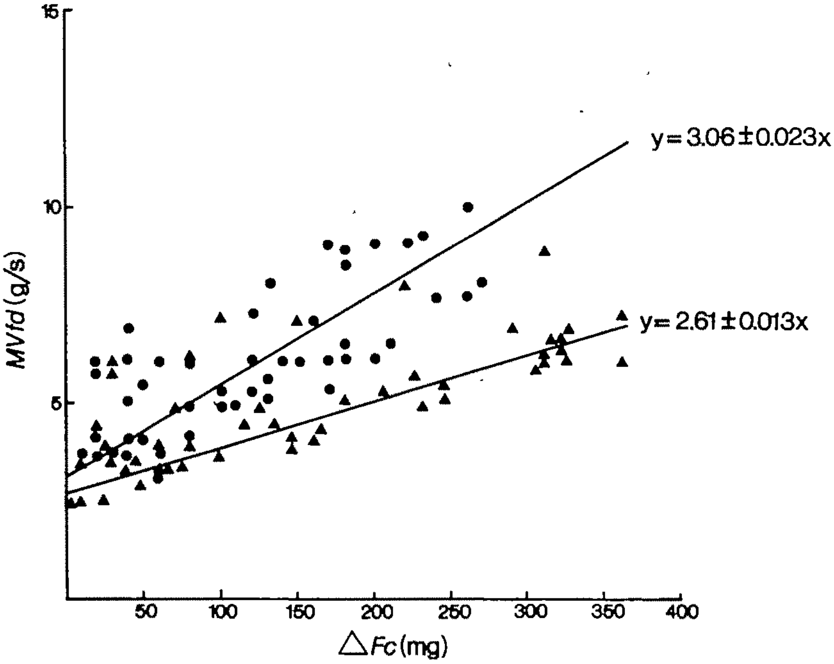

Figure 7 Correlation between inotropic effect and maximum velocity of force development in preparations treated with phenylephrine and driven either at 1 Hz (▲) or at 2.5 Hz (●). Incubation temperature: 30° C.

phenylephrine at low and high stimulation frequencies. Figure 6 shows the recordings of the concentration-inotropic effect curves obtained in the same preparation treated with phenylephrine and driven either at 1 Hz (a) or at 2.5 Hz (b). The difference in the endpoint of the curves obtained at the two frequencies is evident. Moreover at 1 Hz the inotropic effect of the lower concentrations of the amine was due to an increase in the time to peak tension without any change of *MVtd*; an increase of this parameter is evident only at the higher concentrations, associated with a progressive shortening of the time to peak. It is noteworthy that the linear correlation existing between the phenylephrine-induced ΔFc and *MVfd* ($r = 0.861$) at 1 Hz shows a regression coefficient (0.013) significantly lower ($P < 0.001$) than the one observed at 2.5 Hz (Figure 7).

Discussion

In preparations driven at 2.5 Hz both phenylephrine and isoprenaline induce an increase of contractile force by a rise of *MVfd*, which can be considered an index of the intensity of the active state (Panefsky, 1971). However, a distinct difference concerning the relaxation time can be found between the inotropic effects of the two amines. This implies that the cellular mechanisms involved in the effects of the α- and β-adrenoceptor agonists are not the same.

With regard to the cellular mechanism of the inotropic actions, the interaction of the amines with compound D_{600} is important. The increase of cardiac contractility induced by isoprenaline, as well as by other catecholamines is ascribed to an increase of calcium influx probably mediated by an increase in the level of cellular cyclic AMP (Kukovetz & Poch, 1972). Our findings confirm that isoprenaline, acting as a promoter of calcium transmembrane influx is able to counteract the negative inotropic effect of D_{600} (Fleckenstein, 1971). On the basis of its antagonistic action against D_{600}, phenylephrine seems to be a weak promoter of calcium transmembrane influx. This is in agreement with the observed lack of effect of phenylephrine on cyclic AMP levels (Benfey, 1971; Benfey & Carolin, 1971).

Moreover the inotropic effect of phenylephrine shows an evident frequency-dependence: the net increase of contractile tension induced by the same concentration of the amine is significantly higher at 1 Hz than at 2.5 Hz. Despite the increased contractility, the slope of the linear correlation between ΔFc and *MVfd* is significantly lower at 1 Hz than at 2.5 Hz; this implies that the increased intensity of the active state is of less importance at 1 Hz than at 2.5 Hz and that some other factor must play a role in the inotropic response at the lower frequency of stimulation. The suggestion can be advanced that this factor is a prolongation of the active state, because an increase of time to peak force is seen in preparations treated with phenylephrine at the lower but not at the higher stimulation rate.

In the light of these observations a correlation can be attempted between mechanical and electrophysiological effects of phenylephrine on cardiac muscle. (Ledda & Marchetti, 1971; Ledda *et al.*, 1971.) Studies on correlation between electrical and mechanical events show that the prolongation of the action potential duration tends to increase the time to peak force thus increasing developed tension, either in mammalian (Morad & Trautwein, 1968; Braveny & Sumbera, 1970) or in amphibian heart (Vassort & Rougier, 1972). If the prolongation of the plateau phase of the action potential is an important factor in the mechanism of the inotropic action of phenylephrine, it is possible to explain the smaller effect of the amine at the higher frequency of stimulation on the basis of the well known inverse relationship between action potential duration and rate.

With regard to the two different mechanisms which seem to be involved in the inotropic effect of phenylephrine at the lower frequency, namely an increase in time to peak tension at the lower concentrations and an increase in maximum velocity of force development at the higher, it is necessary to bear in mind that phenylephrine is a weak agonist of β-adrenoceptors (Furchgott, 1972). Therefore it is conceivable that the isoprenaline-like effect (i.e. increase of *MVfd*) of the higher concentrations of the amine could be due to activation of β-receptors, which seem to be dominant in the heart. This interpretation is in agreement with the observation of Govier (1968) and Parr & Urquilla (1972) that the positive inotropic effects of high doses of phenylephrine are antagonized by β-adrenoceptor antagonists.

We wish to thank Professor A. Giotti for his helpful suggestions during the work and for his valuable comments on the manuscript. This work was partly supported by a grant from the Italian Research Council.

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(Received June 25, 1974.
Revised August 26, 1974.)

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INTERACTION OF COMPETITIVE ANTAGONISTS: THE ANTI-CURARE ACTION OF HEXAMETHONIUM AND OTHER ANTAGONISTS AT THE SKELETAL NEUROMUSCULAR JUNCTION

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1 In the rat isolated diaphragm preparation hexamethonium and other low potency competitive antagonists of acetylcholine (ACh), including gallamine and hyoscine butylbromide, reverse block by the potent antagonists tubocurarine, pancuronium and alcuronium.

2 In the presence of tubocurarine, hexamethonium increases the amplitude of the end-plate potential without increasing the quantal content. It enhances the response to ACh applied iontophoretically to the end-plate but does not enhance the response to ACh applied in the bath.

3 The anti-curare effect of hexamethonium is abolished in the diaphragm of the rat, guinea-pig and mouse by inhibitors of acetylcholinesterase. The effect is not observed in the indirectly stimulated toad sartorius muscle.

4 The effect is explained if tubocurarine does not dissociate appreciably in the time taken for ACh to achieve high occupancy of receptors, so that a fraction of receptors is completely excluded from occupation by ACh. Equilibration with hexamethonium reduces the fraction excluded by tubocurarine and the transmitter now competes with hexamethonium for more receptors and produces a larger response.

5 On the basis of this explanation the half-time for dissociation of tubocurarine must be about 1 millisecond. It follows that tubocurarine does not act competitively with ACh at synapses when transmitter action is sufficiently brief, and that its binding to the receptor is probably diffusion-limited.

Introduction

It appears self-evident that block caused by one competitive antagonist should be enhanced by another. However, it has been demonstrated (Stephenson & Ginsborg, 1969) on the basis of the mass-law description of drug-receptor interaction that this need not apply when one antagonist does not dissociate appreciably from the receptors during application of the agonist, and the other is able to compete with the agonist for the unoccupied receptors. Stephenson & Ginsborg (1969) suggested that these conditions might be found during block of transmission at the skeletal neuromuscular junction. Ferry & Marshall (1971) have described an anti-curare action of hexamethonium in the rat isolated phrenic nerve-diaphragm preparation and have subsequently (Ferry & Marshall, 1973) interpreted the phenomenon in terms of the Stephenson-Ginsborg hypothesis.

In this paper we describe experiments which confirm the observations of Ferry & Marshall, and others which indicate that the anti-curare action of hexamethonium is but one example of a more generally observable and seemingly anomalous interaction between competitive antagonists. We have found, as predicted from the mass-law theory, that low potency competitive antagonists including gallamine and hyoscine butylbromide can reverse block by high potency antagonists. The results shed light on the kinetics of the drug-receptor interaction in relation to the time course of transmitter action.

Preliminary reports of this work were given to the Physiological Society of New Zealand and to the Australian Physiological and Pharmacological Society.

Methods

Rat diaphragm

Isolated phrenic nerve-diaphragm preparations from albino rats weighing 100-200 g were maintained at 32° C (for intracellular recordings) or at 37° C (for mechanical recordings) in a physiological solution of composition (mmol/l)· Na^+ 143.5, K^+ 5.9, Ca^{2+} 2.0, Mg^{2+} 1.0, Cl^- 127.2, SO_4^{2-} 1.0, $H_2PO_4^-$ 1.2, HCO_3^- 25.0, glucose 11, and insulin (1 iu/l); equilibrated with 95% O_2 and 5% CO_2. Intracellular recordings of membrane potentials were made with glass capillary microelectrodes filled with KCl (3 mol/l) directly coupled through a W-P Instruments M4A electrometer to an oscilloscope and chart recorder.

End-plate potentials evoked by supramaximal stimulation of the phrenic nerve at 1 Hz were recorded for 4 min from cells whose resting potentials were greater than 50 mV and stable to within 3 mV. Solutions contained (+)-tubocurarine chloride in a concentration of 1.7×10^{-6} mol/l with or without hexamethonium in a concentration of 4×10^{-4} mol/litre. The methosulphate of hexamethonium was used in preference to the bromide which depolarized end-plates, presumably because our samples were contaminated.

Quantal contents of end-plate potentials were calculated from the signal variance corrected for noise (Hubbard, Llinás & Quastel, 1969)· r.m.s. noise was estimated from the film record as one quarter of the peak-to-peak noise. This assumes that noise was random, and that limits fitted by eye to the baseline noise include 95% (±2 s.d.) of the total noise. Records were analysed only if the observed variance was at least 50% greater than the noise variance.

In experiments with acetylcholine (ACh) applied in the bath the depolarization was taken as the difference between the baseline value (steady within 1 mV) and the new steady value which was usually established within 2 min of the start of depolarization. Results were rejected if the depolarization due to ACh could not be reversed by washing.

In the iontophoretic experiment performed by Dr A.J. Harris, the micropipette containing ACh was placed close to the end-plate, and ACh was ejected using a 5 ms current pulse. This experiment was performed at room temperature (22° C).

To test for anti-curare effects of low-potency antagonists, the twitch amplitude of indirectly stimulated muscle preparations was recorded isometrically at a resting tension of 0.05 newton (N) and stimulus frequency of 0.067 Hz. Sufficient tubocurarine was added to the 25 ml bath to reduce the twitch amplitude to a steady level below 50% of the control. The effect of successively adding increments equal to a tenth of the estimated 50% blocking dose of the weak antagonist was then observed. Antagonism of block by alcuronium and pancuronium was observed in the same way. Relative potencies of drugs were determined by estimating the approximate 50% blocking doses (ED_{50}'s) in one diaphragm.

Guinea-pig and mouse diaphragms

Twitch amplitudes of isolated, indirectly stimulated, left hemi-diaphragms of two albino guinea-pigs (150-200 g) and four adult mice were recorded isometrically: resting tensions were 0.05 and 0.02 N tension respectively. The physiological salt solution (37° C) was the same as that used for the rat diaphragm.

Toad muscle

Isolated, innervated sartorius muscles of the toad (*Bufo marinus*) were maintained at room temperature (22° C) in a solution of composition (mmol/l) Na^+ 112, K^+ 2.0, Ca^{2+} 1.8, Cl^- 117.6, HCO_3^- 2.4, $H_2PO_4^-$ 3.0, glucose 11. Muscle contractions were recorded isometrically at a tension of 0.1 N at frequencies of either 0.067 Hz or 1 Hz.

Materials

Drugs used were: (+)-tubocurarine chloride (McGaw Ethicals, Auckland), alcuronium chloride (Roche), pancuronium bromide (Organon), gallamine triethiodide (M. & B.), hexamethonium dibromide (M. & B.), hexamethonium methosulphate, azamethonium (Ciba), hexamethylene bisethyldimethylammonium bromide (Gaplegin, M. & B.), hyoscine butylbromide (Boehringer Ingelheim), atropine sulphate (B.D.H.), homatropine hydrochloride (MacFarlane Smith, Edinburgh), chlorisondamine (Ciba), neostigmine methylsulphate (Roche), edrophonium chloride (Roche), ecothiopate iodide (Ayerst Lab. Inc., N.Y.), physostigmine salicylate (B.D.H.).

Results

Anti-curare effect of hexamethonium

Rat diaphragm We agree with the conclusion of Ferry & Marshall (1971; 1973) that the anti-curare action of hexamethonium in the rat diaphragm (see Figure 4a) is not presynaptic, and that the postsynaptic action is not due to inhibition of cholinesterase or to membrane depolarization. Thus we observed, as they did, that at a tubocurarine concentration of 1.7×10^{-6} mol/l, hexamethonium (4×10^{-4} mol/l) approximately doubled the amplitude of the intracellularly recorded end-plate potential without increasing the quantal content (Table 1). Hexamethonium

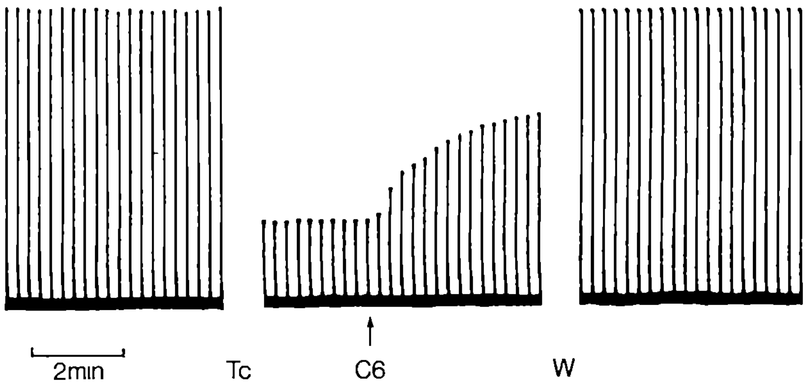

Figure 1 Anti-curare effect of hexamethonium in the indirectly stimulated guinea-pig diaphragm preparation. Tubocurarine (Tc) (3×10^{-6} mol/l) present in centre block. Hexamethonium (C6) (4×10^{-4} mol/l) added at arrow. Forty min elapsed between washout of the antagonists and the final record. Control twitch tension approximately 0.25 N. Recording isometric.

did not increase the duration of the end-plate potential or affect the resting membrane potential recorded at the end-plate in the presence of tubocurarine. Increases in the concentration of hexamethonium beyond 4×10^{-4} mol/l generally caused little or no further increase in the amplitude of the end-plate potential and eventually caused greater block: acting on its own, hexamethonium causes 50% block of transmission in the rat diaphragm at a concentration of about 4×10^{-3} mol/l (Blackman, unpublished observations).

We have confirmed also that hexamethonium does not antagonize partial block of transmission by Mg^{2+}. This is further evidence that hexamethonium at least in concentrations up to 10^{-3} mol/l does not inhibit cholinesterase in the diaphragm, and is evidence also that hexamethonium has no effect on the passive properties of the end-plate membrane that would account for its anti-curare effect. Its action appears to depend on an interaction with tubocurarine at receptor level.

Diaphragm of other mammals The anti-curare action of hexamethonium was observed in the isolated hemi-diaphragms of two guinea-pigs (Figure 1). Similar results were observed in diaphragms from four mice. The effect in both species was characterized by a dependence on concentration, similar to that in the rat diaphragm.

Effect in the amphibian Given that the chief features of transmission at mammalian and amphibian neuromuscular junctions are fundamentally similar, we expected to observe the anticurare effect of hexamethonium at the toad sartorius neuromuscular junction. However, in this preparation, hexamethonium exhibited no anti-curare effect at concentrations below 10^{-4} mol/l; at higher concentrations it simply enhanced the block by tubocurarine.

Anti-curare effect of other competitive antagonists

Table 2 demonstrates, that a range of weak antagonists including hexamethonium partially

Table 1 Effect of hexamethonium on the mean amplitude and quantal content of end-plate potentials (e.p.p.) in the curarized diaphragm of the rat

Treatment	*No. of cells*	*Mean* e.p.p. amplitude (mV)*	*Mean quantal content*
Tubocurarine	8	0.55 ± 0.05	168 ± 36
Tubocurarine plus hexamethonium	12	0.93 ± 0.11	128 ± 14

* Each amplitude is the mean of 180 responses measured in one cell. Concentrations (mol/l): tubocurarine, 1.7×10^{-6}; hexamethonium, 4.0×10^{-4}. Values given are means ± s.e. mean.

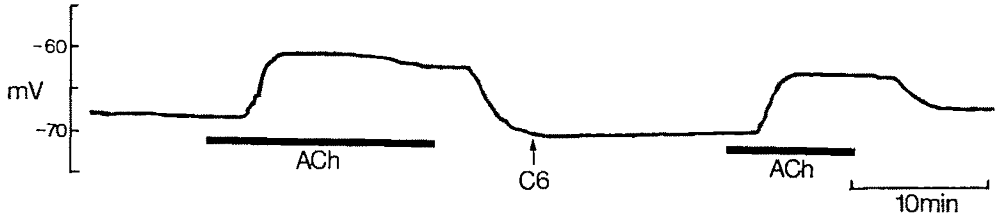

Figure 2 Failure of hexamethonium (C6: 4.0×10^{-4} mol/l) to enhance the depolarization of a muscle fibre by bath-applied acetylcholine (ACh: 4.0×10^{-3} mol/l) in the presence of tubocurarine (1.7×10^{-6} mol/l). Intracellular recording.

reversed neuromuscular block produced by tubocurarine, pancuronium, and alcuronium, all of which are potent competitive antagonists. These anti-curare actions were observed at a concentration of each weak antagonist generally about 10% of its ED_{50} for neuromuscular block. Increasing this dose towards the ED_{50} ultimately enhanced block.

Hyoscine butylbromide exhibited a weak anti-curare effect against tubocurarine and alcuronium, but not against pancuronium. The antagonism of tubocurarine and alcuronium was not observed in all diaphragm preparations.

Effect of other drugs

It follows from the hypothesis that the anti-curare effect should be confined to low-potency competitive antagonists. It was of interest, therefore, to test substances known not to be competitive antagonists at the neuromuscular junction. Chlorisondamine was chosen first because it is a bis-onium compound like hexamethonium, and of similar potency, but with a non-competitive action (van Rossum & Ariëns, 1959; Trendelenburg, 1961). It had no anti-curare effect in the rat diaphragm at any concentration (Table 2). Similarly atropine had no anti-curare effect (Table 2): its action at the neuromuscular junction has recently been shown to be non-competitive in the sense that it affects the elementary conductance change underlying the end-plate current (Katz & Miledi, 1973b). Homatropine, also, had no anti-curare effect.

Dependence on method of application of acetylcholine

The explanation of the anticurare action of hexamethonium favoured by Ferry & Marshall and by ourselves is based on the mass-law description of drug-receptor interaction. It assumes that the 'slow' antagonist does not dissociate appreciably in the time taken for the agonist to achieve high occupancy of receptors. The effect should not

Table 2 Effects in the rat diaphragm of weak antagonists of acetylcholine on partial neuromuscular block by potent antagonists

	Tubocurarine	*Pancuronium*	*Alcuronium*	*Approx. equipotent molar ratio*
Gallamine	+	+	+	86
Gaplegin*	++	++	++	3000
Hexamethonium	++	++	++	5000
Azamethonium	++	++	++	6300
Atropine	—	—	—	2000[a]
Homatropine	—			
Hyoscine butylbromide	+	—	+	3200
Chlorisondamine	—			

Substantial reversal (see Figure 4a) is expressed as ++; a lesser degree of reversal is expressed as + and no reversal as —. Each observation was made in two or more diaphragms. Equipotent molar ratios (relative to tubocurarine) were measured in one diaphragm only. Stimulus frequency 0.067 Hz.

[a] Beránek & Vyskocil (1967).

* Hexamethylene bisethyldimethylammonium bromide.

occur, therefore, when the agonist is applied slowly, as from the bath.

Bath application Figure 2 illustrates the result of an experiment in which the end-plate response to ACh applied in the bath was recorded from a single muscle cell before and after adding hexamethonium to the curarized diaphragm. Cells were accepted only when there was no decline in response to ACh over the first 5 min of application. In the 8 cells in which this experiment was performed, hexamethonium produced no consistent change in the depolarizing response to ACh (Table 3). In another set of experiments on the curarized diaphragm in which similar drug concentrations were used, the mean depolarization produced by bath-applied ACh in 13 cells was not significantly different from the depolarization produced in 13 other cells when hexamethonium was present as well (Table 4). It is evident from these 'equilibrium' experiments that hexamethonium does not potentiate the response to ACh in the curarized diaphragm when ACh is added slowly from the bath.

Iontophoretic application In an experiment kindly performed by Dr A.J. Harris, ACh was applied by iontophoresis to the end-plates of two cells of a curarized rat diaphragm preparation before and after adding hexamethonium. The amplitude of the ACh potential evoked by a 5 ms rectangular ejection pulse increased when hexamethonium was present and returned to its original level when hexamethonium was withdrawn (Figure 3). This result, which incidentally confirms that the mechanism of the anti-curare action of hexamethonium is postsynaptic, suggests that ACh applied from a micropipette can achieve high occupancy of receptors before appreciable dissociation of tubocurarine occurs. However, we did not determine the effect of applying ACh at different rates, or attempt to test other explanations of the anticurare effect using this technique.

Influence of synaptic factors

The result in the amphibian suggests that the occurrence of the anticurare effect of hexamethonium is critically dependent on synaptic

Table 3 End-plate depolarization of curarized rat diaphragm by bath-applied acetylcholine before and after adding hexamethonium

Cell number	*Resting membrane potential (mV)*	*Without added hexamethonium*	*With added hexamethonium*	*Difference*
1	68	6.0	6.0	0.0
2	70	2.5	3.0	+0.5
3	70	3.0	2.5	–0.5
4	60	2.3	3.5	+1.2
5	65	5.0	5.5	+0.5
6	64	1.2	3.0	+1.8
7	56	3.0	2.5	–0.5
8	60	2.3	2.0	–0.3
Mean		3.16	3.50	+0.30*

Drug concentrations (mol/l): acetylcholine 4 x 10^{-3}; tubocurarine 1.7 x 10^{-6}; hexamethonium 4 x 10^{-4}. Hexamethonium was added in reverse order in cells 2, 4 and 6.
* Mean difference not significant ($P > 0.05$).

Table 4 End-plate depolarization of curarized rat diaphragm by bath-applied acetylcholine in the absence and in the presence of hexamethonium

Treatment	*No. of cells*	*Mean depolarization (mV)*
Tubocurarine	13	2.13 (± 0.39)
Tubocurarine plus hexamethonium	13	1.92 (± 0.23)
	Difference	0.21*

Values given are means ± s.e. mean.
Concentration (mol/l): acetylcholine, 4.0 x 10^{-3}; tubocurarine, 1.7 x 10^{-6}; hexamethonium, 4.0 x 10^{-4}.
* Not significant ($P > 0.05$).

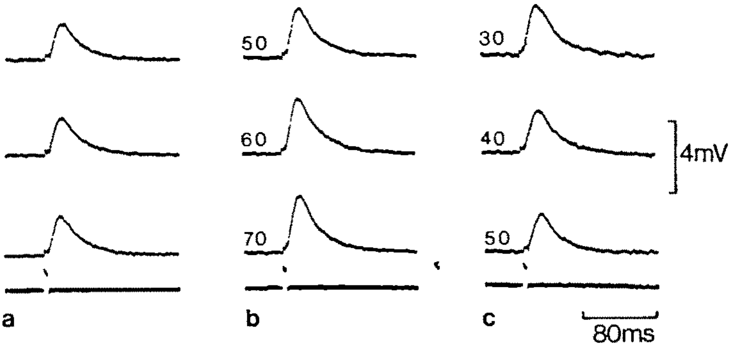

Figure 3 Effect in a curarized rat diaphragm preparation of hexamethonium (4.0×10^{-4} mol/l) on the potential produced by iontophoretic application of acetylcholine. Numbers denote time in min after addition (between (a) and (b)) or withdrawal (between (b) and (c)) of hexamethonium. Tubocurarine: 1.7×10^{-6} mol/litre.

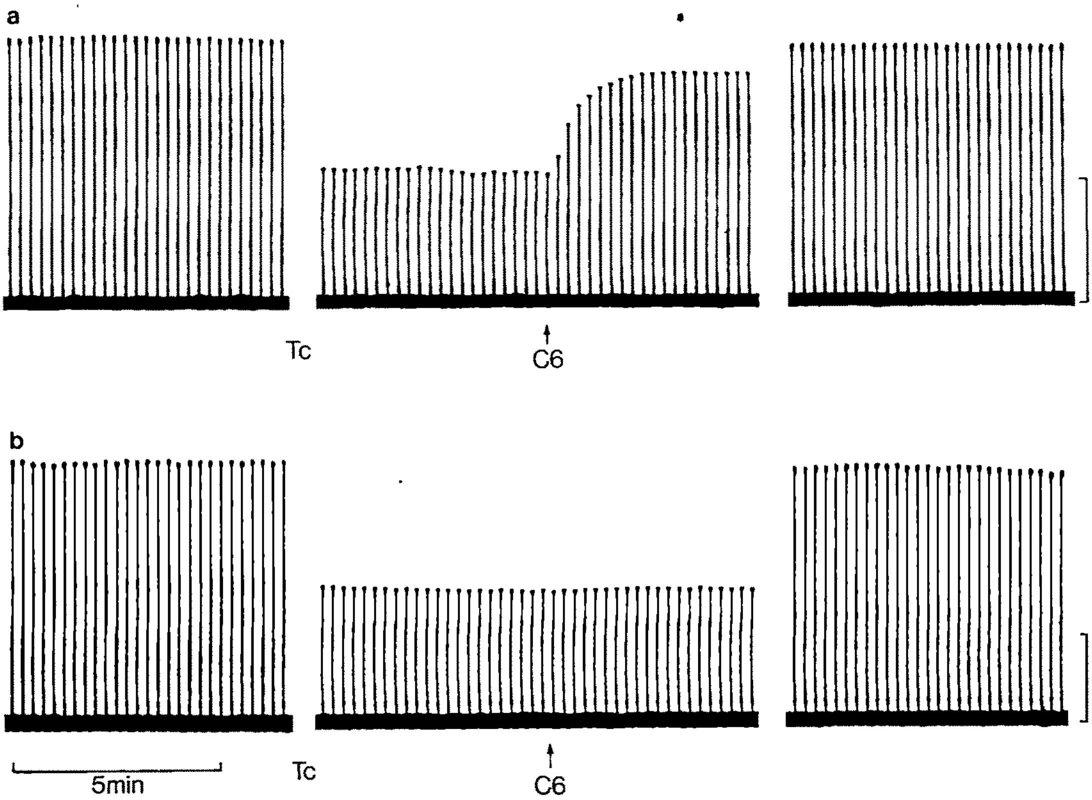

Figure 4 The effect of hexamethonium (C6: 4.0×10^{-4} mol/l) on twitch tension of an indirectly stimulated curarized rat diaphragm in the absence (a) and in the presence (b) of neostigmine (3×10^{-6} mol/litre). At least 20 min was allowed for equilibration with tubocurarine. (Tc: approx. 1×10^{-6} mol/litre). Isometric recording, vertical calibration 0.1 N.

factors. We have considered three: temperature, ACh concentration, and the time course of events.

Temperature The anticurare effect of hexamethonium was observed in the rat diaphragm at temperatures ranging from 24° C to 37° C, but could not be induced in the toad sartorius preparation by raising the temperature from 20° C to 37° C.

Acetylcholine concentration Variation of stimulus frequency (from 0.06 to 2.0 Hz) and of calcium levels (from 2 to 8 x 10^{-3} mol/l) did not affect the anticurare action of hexamethonium in the rat diaphragm. We conclude that the effect is not critically dependent on the synaptic concentration of ACh during transmission.

Time course of events We attempted to modify the time course of synaptic events associated with a given degree of block in the rat diaphragm by simultaneously inhibiting acetylcholinesterase activity and adding tubocurarine to maintain the level of block. When this was done with neostigmine (3 x 10^{-6} mol/l) the anticurare effect of hexamethonium was abolished (Figure 4b). Similarly, neostigmine abolished the effect in the guinea-pig and mouse preparations. Edrophonium (5 x 10^{-6} mol/l), ecothiopate (0.5 x 10^{-6} mol/l) and physostigmine (5 x 10^{-6} mol/l) also abolished the effect in the rat diaphragm.

In addition, neostigmine (3 x 10^{-6} mol/l) abolished the anti-curare effects of hexamethylene bisethyldimethylammonium, gallamine, and hyoscine butylbromide, and reduced that of azamethonium.

Discussion

It is evident from our results that the anti-curare effect of hexamethonium is but one example of a general phenomenon which is a consequence of the kinetics of the drug-receptor interaction. The phenomenon is confined to competitive antagonists and conforms qualitatively with the expectations of the hypothesis derived by Stephenson & Ginsborg (1969) from the mass-law description of drug-receptor interaction. These authors have shown that a rapidly dissociating antagonist can reverse block by a slowly dissociating antagonist acting on the same receptors, provided exposure to the agonist is so brief that little dissociation of the slow antagonist occurs during its action: the slow antagonist thus completely excludes a fraction of receptors from occupation by the agonist. Equilibration with the rapidly dissociating antagonist reduces the fraction excluded and the agonist then competes with the fast antagonist for more receptors. Block is reversed.

In the terminology of Stephenson & Ginsborg (1969) the conditions for reversal can be stated as follows. Let y_b be the fraction of receptors occupied by the agonist a in the presence of the slow antagonist b alone, and let y_{bc} be the fraction occupied by the agonist in the presence of both the slow antagonist b and the fast antagonist c. Given that b dissociates to a negligible extent during the application of the agonist a, but that c dissociates sufficiently rapidly to equilibrate with the agonist, it can be shown that

$$y_b = Y(1-Z)$$

and

$$y_{bc} = \frac{Y(1-X)(1-Z)}{(1-XY)(1-XZ)}$$

where Y = fraction of receptors occupied by agonist acting alone
X = fraction occupied by fast antagonist acting alone
Z = fraction occupied by slow antagonist acting alone

The condition for reversal by c of block by b is therefore

$$\frac{y_{bc}}{y_b} = \frac{1-X}{(1-XY)(1-XZ)} > 1$$

a condition which we presume occurs in our experiments in the rat diaphragm. In the diaphragm, the transmitter, ACh, acts rapidly and within a millisecond is removed by hydrolysis and diffusion (see Hubbard, 1973). It is thus likely, as Stephenson & Ginsborg (1969) have observed, to fulfil the requirements of the agonist in the above scheme.

Tubocurarine almost certainly fulfils the requirements for the slow antagonist. It has been shown to be a competitive antagonist of ACh over a wide range of concentrations at the skeletal neuromuscular junction of the frog (Jenkinson, 1960) and of the guinea-pig lumbrical (Waud, Cheng & Waud, 1973) and diaphragm muscles (Lu, 1970). Its affinity constant has not been measured in the rat diaphragm but is likely to be close to the value of 9 x 10^6 l/mol measured in guinea-pig diaphragm and lumbrical muscles. Given this affinity constant and an upper limit of 2.5 x 10^9 l mol^{-1} s^{-1} for the forward rate constant of the drug-receptor interaction (see Burgen, 1966), the half-time for dissociation of tubocurarine from the receptor must be at least 3 ms or greater.

Similar arguments can be applied to pancuronium, whose affinity constant is even larger than that of tubocurarine (Waud *et al.,* 1973), and to alcuronium which is about as potent as tubocurarine. Since the end-plate potential in the rat diaphragm has a time-to-peak usually less than 1 ms, these three antagonists should dissociate only slightly, if at all, during transmission: their action should be effectively irreversible. There is indeed evidence that tubocurarine dissociates only slightly during neuromuscular transmission in the mammal (Paton & Waud, 1967; Kruckenberg & Bauer, 1971).

By contrast, the dissociation of hexamethonium from receptors could approach completion during transmission. Hexamethonium appears to act as a competitive antagonist of ACh with an affinity constant of about 2.5×10^4 l/mol (Ariëns, van Rossum & Simonis, 1956) Its corresponding half-dissociation time could be about one hundredth that of tubocurarine, that is 10 μs or greater. A large fraction of hexamethonium-receptor complexes may therefore dissociate during brief application of the transmitter, and hexamethonium may thus fulfil the requirements for the fast antagonist *c*. Similar arguments apply to the other low-potency competitive antagonists tested.

Antagonists which dissociate less rapidly than hexamethonium should have less anti-curare effect. Thus the anti-curare effect of gallamine was weaker than that of hexamethonium. It must be supposed that the dissociation rate of gallamine is not slow enough to allow hexamethonium to antagonize its blocking action (Ferry & Marshall, 1971), although the theory does not exclude the possibility of a compound acting as both a fast and slow antagonist.

Our argument, so far, supports the view that the Stephenson-Ginsborg hypothesis explains the anti-curare effect of hexamethonium and related observations. We believe, however, that the theory in its given form provides no more than a general description of events, for certain assumptions implicit in the hypothesis may not be fulfilled at the neuromuscular junction.

The mathematical formulation assumes that the agonist is applied uniformly to the receptor population and that it equilibrates with the receptors. However, at the skeletal neuromuscular junction, application of ACh is unlikely to be uniform, for it is released asynchronously and from a number of discrete sites (Dreyer, Peper, Akert, Sandri & Moor, 1973; Hubbard, 1973). Equilibration may not occur either for if, as has been argued, ACh has an affinity constant for the receptors of 10^6 l/mol or greater (Eldefrawi, 1974), it can be expected to dissociate with a half-time of at least 0.3 millisecond. This is a substantial fraction of its lifetime in the cleft and must preclude it from coming to equilibrium. As it happens, this is of no consequence to our explanation, which requires only that dissociation of hexamethonium from receptors should take place rapidly enough to allow acetylcholine to achieve high occupancy before tubocurarine begins to dissociate.

It follows that the anti-curare effect should be critically dependent on the rate of occupation of receptors by the agonist. The rate of occupation of receptors by ACh released from the nerve terminal is unfortunately not easily controlled experimentally, for release is in quanta from discrete sites within the cleft. Some control can be achieved, however, by applying ACh by diffusion from outside the cleft. Thus we have observed that the effect does not occur in the rat diaphragm when ACh is applied from the bath, but does occur when ACh is applied by iontophoresis. We assume in the latter case that the concentration gradient is steep enough to achieve high occupancy of successive populations of receptors before tubocurarine dissociates appreciably. By contrast, the rate of application of ACh from the bath is limited by the time for mixing and must be very much slower.

We have also taken as evidence of the dependence of the effect on rate of occupation of receptors our finding that anticholinesterase agents abolish the anti-curare effect of hexamethonium. Our argument runs as follows. When the esterase is inhibited, more tubocurarine must be present to maintain the same degree of block. Under these circumstances, although the amplitude of the response is the same as that before the cholinesterase was inhibited, the time course of the response to ACh is prolonged. In particular, the time-to-peak of the response is longer, which implies that the rate of occupation of receptors is slower. There is evidence also that when cholinesterase is inhibited, ACh diffuses laterally from its presynaptic release sites to more distant receptors (Negrete, del Castillo & Escobar, 1972) and activates receptors in succession before being removed by diffusion from the synaptic cleft (Katz & Miledi, 1973a). Tubocurarine should, therefore, dissociate to an appreciable extent before ACh achieves adequate occupancy of receptors. In consequence, hexamethonium will have no anti-curare effect. It may be noted in passing that the anti-curare effects of the other weak antagonists tested in our experiments were also abolished by neostigmine. This is consistent with our belief that they share with hexamethonium the same mechanism of anti-curare action.

Two factors may conspire to ensure that hexamethonium should have no anti-curare effect in the toad sartorius preparation: the rate of occupation of receptors by the transmitter may be slower in the amphibian than in the mammal, and tubocurarine may dissociate more rapidly from receptors in the amphibian than in the mammal. One measure of the rate of occupation of receptors is the rise time of the end-plate current. As it happens, there appears to be little difference at the end-plates of frog and rat in the time-to-peak of the synaptic current due to a single quantum of ACh (150-300 μs: Gage & Armstrong, 1968). However, the time-to-peak of the end-plate current will depend also on the degree of asynchrony in release of quanta of transmitter: it would be interesting to compare the rise time of the end-plate current in the rat diaphragm with that in the toad sartorius muscle.

In addition, tubocurarine appears to dissociate more rapidly from amphibian than from mammalian muscle receptors. Thus as an antagonist of iontophoretically applied ACh at the motor end-plate, tubocurarine is 20 times less effective in the frog than in the rat (Beránek & Vyskočil, 1967, 1968). Also, the affinity constant in the frog is three times lower than in the guinea-pig (Jenkinson, 1960; Lu, 1970; Bowen, 1972; Waud *et al.*, 1973) and in the toad rectus abdominis muscle is lower by a factor of 15 or more (Blackman & Milne, unpublished results). We conclude, therefore, that anomalous interaction of hexamethonium and tubocurarine does not occur in the amphibian because, for one or both of the reasons discussed above, tubocurarine is able to dissociate significantly from receptors during application of the transmitter.

Finally, our results allow calculation of the half-time for dissociation of tubocurarine from receptors in the rat diaphragm If tubocurarine remains bound to receptors during the rise-time of the end-plate potential (about 0.7 ms) but dissociates significantly when the rise-time is prolonged by inhibition of cholinesterase, we can conclude that the half-dissociation time for tubocurarine is not less than 0.7 ms and is not greater than the time-to-peak of the prolonged end-plate potential. It is therefore likely to be about 1 millisecond. Assuming this value of half-time for dissociation and an affinity constant of 9×10^6 l/mol, it follows that the forward rate constant for binding to receptors is about 6×10^9 l mol^{-1} s^{-1}. This compares with the theoretical maximum rate constant for diffusion-limited binding (2.5×10^9 l mol^{-1} s^{-1}. Burgen, 1966). This is a satisfactory consequence of our explanation of the anti-curare effect of hexamethonium. It incidentally implies that binding of tubocurarine to the ACh receptors of mammalian diaphragm muscle is diffusion limited.

Note added in proof The reader is referred to a full account of the Stephenson-Ginsborg hypothesis which has appeared since our paper was written (GINSBORG, B.L. & STEPHENSON, R.P. (1974). On the simultaneous action of two competitive antagonists. *Br. J. Pharmac.*, 51, 287-300).

This work was supported by a grant from the Medical Research Council of New Zealand.

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THE ACTIONS OF DIHYDROXYPHENYLALANINE AND DIHYDROXYPHENYLSERINE ON THE SLEEP-WAKEFULNESS CYCLE OF THE RAT AFTER PERIPHERAL DECARBOXYLASE INHIBITION

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1 The actions of dihydroxyphenylalanine (DOPA) and dihydroxyphenylserine (DOPS) were assessed on the sleep-wakefulness cycle of male Wistar rats.

2 In comparative studies the extracerebral decarboxylase was inhibited with serine-trihydroxybenzylhydrazide (RO 4-4602) before injection of DOPA or DOPS.

3 DOPA (80-160 mg/kg, i.p.) with or without previous inhibition of the peripheral decarboxylase gave rise to an initial significant increase of slow wave activity, which may be related to a release of 5-hydroxytryptamine

4 During the subsequent 8 h sessions, DOPA significantly decreased slow wave sleep and rapid eye movement sleep (REM) and increased wakefulness.

5 DOPS (80-160 mg/kg, i.p.) did not significantly modify the sleep-wakefulness cycle apart from a decrease of the latency for the first REM episode after 160 mg/kg in the RO 4-4602 pretreated animals.

Introduction

There is at present evidence indicating that catecholamines may be involved in arousal and rapid eye movement sleep (REM), while indoleamines may be related to slow wave sleep (SWS) (Torda, 1968; Jouvet, 1969). Part of this evidence has been obtained by the use of precursors of these amines, including L-dihydroxyphenylalanine (DOPA), DL-dihydroxyphenylserine (DOPS) and L-5-hydroxytryptophan (5-HTP). Since these compounds cross the blood-brain barrier only to a limited extent (Constantinidis, de la Torre, Tissot & Geissbulher, 1969), controversies have arisen as to the origin of the central actions seen after their administration. In this connection, the pronounced autonomic symptoms produced by the injection of DOPA, ascribable to the actions of newly formed catecholamines in the periphery, would be partly responsible for the state of behavioural arousal simultaneously developed (Butcher & Engel, 1969). There is also controversy as to the action of the precursors on the EEG, especially with respect to DOPA, which has been most extensively used. While some authors describe a cortical desynchronization after low doses of DOPA, others emphasize that desynchronization cannot be observed until doses in the lethal range are approached (Harner & Dorman, 1969; Thut & Rech, 1972).

Discrepancies in results could arise not only because of differences in the species examined, different dose ranges and routes of administration, but also as a result of differences in the time of observation, since the biochemical changes induced by the precursor (Ng, Chase, Colburn & Kopin, 1972) could have a temporal sequence giving rise to different EEG patterns.

There is also uncertainty as to whether the EEG effects are due to dopaminergic and/or noradrenergic mechanisms (Lidbrink, Corrodi, Fuxe & Olson, 1972).

It was our aim to study the actions of two catecholamine precursors upon the EEG and the sleep-wakefulness cycle of the rat, of which one, DOPA, was found to produce a marked increase in brain dopamine (Bartholini, Da Prada & Pletscher, 1968), while the other, DOPS, selectively increased noradrenaline (NA) in animal brain (Blaschko, Burn & Langemann, 1950).

In order to exclude the actions of peripherally formed catecholamines, in some experiments extracerebral decarboxylase was inhibited before the injection of the precursors.

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Methods

Six male Wistar rats weighing 180-200 g were used in the EEG studies. They were housed individually and maintained under controlled environmental conditions, with a cycle of 12 h light-12 h darkness (light cycle was from 9 h 00 min to 21 h 00 min). All the animals were chronically implanted with bipolar Nichrome electrodes (200 μm diameter) in the frontal and occipital cerebral cortices, dorsal hippocampus (A 3.0, L 2.0, H +2.0; König & Klippel, 1963) and neck muscles. All the electrodes were wired to a small connector fixed to the skull with dental acrylic. Histological verification of the position of the electrodes was carried out at the end of the experiments.

Ten days after implantation, when fully recovered, each animal was placed in a dimly lit sound-proof box fitted with a one-way mirror and sleep patterns were recorded with a Grass 7 polygraph. When the animals were fully adapted to their new environment as judged by the consistency of their sleep-wakefulness cycles, solvent and drug administration were started. All records were scored for waking, SWS and REM, according to standard criteria.

Three sets of experiments were carried out in a random order:

Experiment 1 DOPA and DOPS were studied at 2 dose levels, 80 and 160 mg/kg.

Experiment 2 A dopa-decarboxylase inhibitor, RO 4-4602 (DL-serine 2-(2,3,4-trihydroxybenzylhydrazide hydrochloride) was administered in a dose of 50 mg/kg, in order to block only extracerebral decarboxylase.

Experiment 3 RO 4-4602 was given followed 30 min later by DOPA or DOPS. During control experiments, the rats received the corresponding volumes of solvent.

Drugs were administered as aqueous solutions or suspensions prepared with distilled water. All the injections were given intraperitoneally and recordings were started immediately after the last injection, although scoring of the 8 h sessions began 20 min later. At least one week was allowed to elapse between experiments and each treatment was given to each animal twice.

Differences in mean values of the different variables were tested by analysis of variance for dependent samples, followed by multiple comparisons using the Scheffé test (Winer, 1962).

Results

Following the administration of DOPA, the animals showed behavioural quiescence. No loss of righting reflex or postural changes could be observed. About 15-20 min after the injection of the catecholamine precursor, marked autonomic symptoms including salivation, piloerection, exophthalmus and increased respiratory rate became evident, lasting about 60-90 minutes. When DOPA was preceded by RO 4-4602, no autonomic signs could be observed, the main effects being stereotyped movements of the head and forepaws which were more pronounced after the dose of 160 mg/kg DOPA and lasted 120-180 minutes.

After DOPA administration with or without the decarboxylase inhibitor, a significant increase of slow wave EEG activity compared with the effect of solvent was seen during the first 20 min of continuous recording (Table 1). Moreover, there

Table 1 Amounts of slow wave (synchronized) and fast (desynchronized) EEG activity during the first 20 min following L-dihydroxyphenylalanine (DOPA) or DL-dihydroxyphenylserine (DOPS) and pretreatment with RO 4-4602

Treatment (mg/kg)	*EEG* *Slow wave activity (s ± s.e. mean)*	 *Fast activity (s ± s.e. mean)*	P*
Control	43 ± 24	1157 ± 24	—
DOPA (80)	481 ± 98	719 ± 98	<0.01
DOPA (160)	373 ± 96	827 ± 96	<0.05
RO (50 + DOPA (80)	300 ± 77	900 ± 77	<0.05
RO (50) + DOPA (160)	330 ± 47	870 ± 47	<0.01
DOPS (80)	196 ± 108	1004 ± 108	NS
DOPS (160)	113 ± 50	1087 ± 50	NS
RO (50) + DOPS (80)	148 ± 87	1052 ± 87	NS
RO (50) + DOPS (160)	268 ± 78	932 ± 78	NS

* All comparisons are with control. Differences in mean values were tested by applying the Scheffé test.

were no significant differences in the amount of slow wave activity following different DOPA treatments.

The EEG synchronization contrasted with the animal's behaviour, standing with open eyes. Subsequently, the EEG pattern shifted to one of continuous desynchronization, the duration varying according to the treatment involved.

Quantitation of the 8 h sessions showed significant drug-induced alterations of the sleep-wakefulness cycle. DOPA (80 mg/kg) increased wakefulness and decreased REM. Wakefulness was augmented mainly during the first 2 h of recording; REM was abolished during the first hour, thereafter increasing steadily and reaching control levels at the fourth hour (Figure 1; Table 2).

Doubling of the dose of DOPA produced a further increase of waking, while SWS and REM time were simultaneously decreased. Waking and SWS attained control values at the fourth hour, while REM returned to control levels 2 h later (Figure 1; Table 2).

Following a dose of 50 mg/kg RO 4-4602, no significant changes could be observed (Table 2). After pretreatment with RO 4-4602, a marked increase of the effects of 80 and 160 mg/kg DOPA on the sleep-wakefulness cycle was observed. Wakefulness became almost continuous during the first 120-180 min and SWS fully recovered only after the fourth and fifth hour following the low and high doses, respectively. REM which was

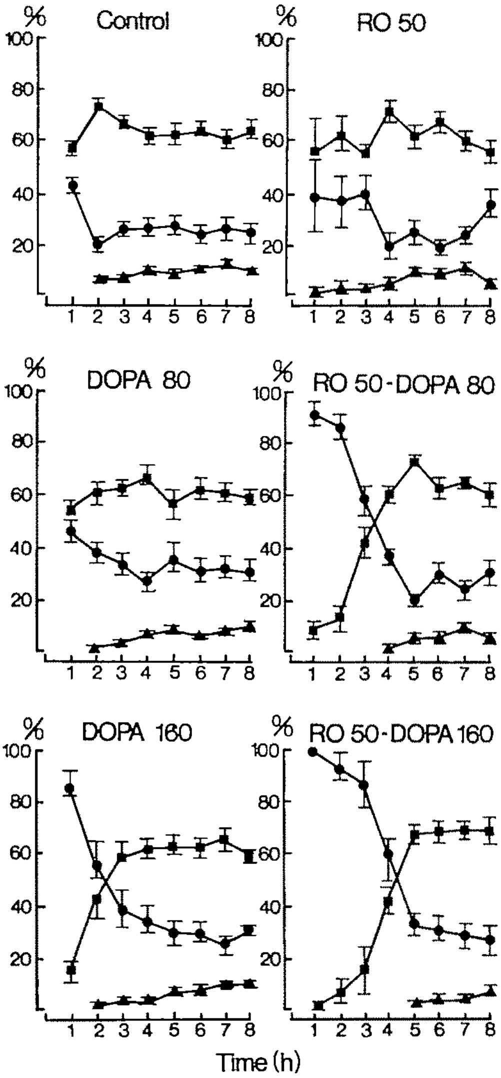

Figure 1 Percentages of waking (●), slow wave sleep (■) and rapid eye movement sleep (▲) during the 8 h sessions after L-dihydroxyphenylalanine (DOPA) administration and pretreatment with RO 4-4602. Abscissae: time in hours. Ordinates: percentage (±s.e. mean) of behavioural stage according to EEG criteria. Doses of drugs in mg/kg.

Table 2 Effects of L-dihydroxyphenylalanine (DOPA) and pretreatment with RO 4-4602 on some variables of the sleep-wakefulness cycle during 8 h sessions starting 20 min after the last injection

Treatment (mg/kg)	*Wakefulness (min ± s.e. mean)*	*Slow wave sleep (min ± s.e. mean)*	*REM sleep (min ± s.e. mean)*
Control	137 ± 14	301 ± 13	42 ± 4
DOPA (80)	167 ± 13	286 ± 12	27 ± 2**
DOPA (160)	215 ± 21**	243 ± 19**	22 ± 3**
RO (50)	145 ± 15	299 ± 13	36 ± 5
RO (50) + DOPA (80)	225 ± 13**	236 ± 9**	19 ± 5**
RO (50) + DOPA (160)	274 ± 14**	199 ± 12**	7 ± 2**

Differences in mean values were compared to control values and tested for significance by applying the Scheffé test.

** $P < 0.01$.

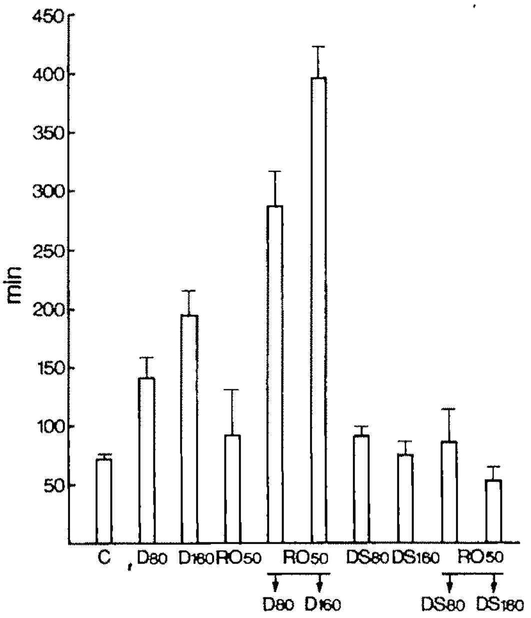

Figure 2 Latencies for the appearance of the first rapid eye movement sleep episode following L-dihydroxyphenylalanine (D) or DL-dihydroxyphenylserine (DS) administration and pretreatment with RO 4-4602 Abscissae: pharmacological treatments The numbers adjacent to the letters indicate mg/kg of drug administered.

almost absent during the first 3-4 h after the smallest dose of the precursor, reached control values at the seventh hour. REM values were below control values throughout the recovery period (Figure 1).

The latency for the appearance of the first REM period was significantly increased after DOPA ($P < 0.01$) in a dose-related manner. There was a further increase in this effect of DOPA after inhibition of peripheral dopa-decarboxylase (Figure 2) although RO 4-4602 alone had no direct effect.

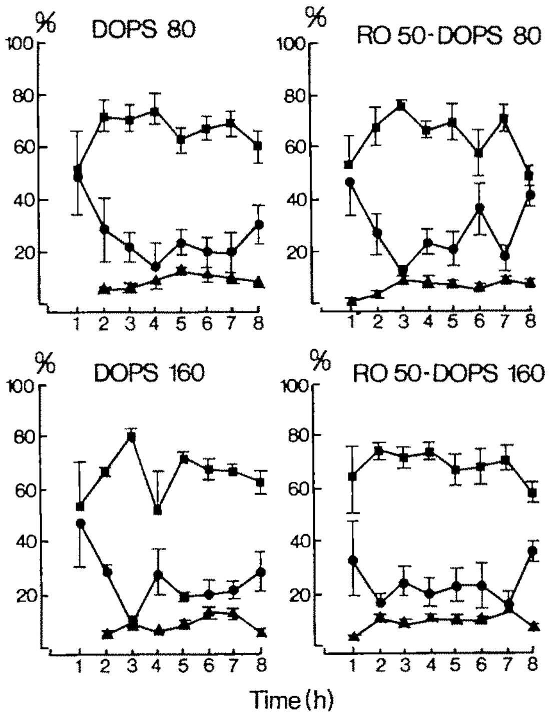

Figure 3 Percentages of waking (●), slow wave sleep (■) and rapid eye movement sleep (▲) during the 8 h sessions after DL-dihydroxyphenylserine (DOPS) administration and pretreatment with RO 4-4602 Abscissae· time in hours Ordinates· percentage (±s e. mean) of behavioural stage according to EEG criteria Doses of drugs in mg/kg.

DOPS in doses of 80 and 160 mg/kg with or without decarboxylase inhibition did not induce behavioural alterations. While asleep, the animals always exhibited normal postures. Although EEG synchronization was augmented during the first 20 min following DOPS, the increase did not reach significance (Table 1). Slow wave sleep values after 160 mg/kg DOPS and 50 mg/kg RO 4-4602 plus

Table 3 Effects of DL-dihydroxyphenylserine (DOPS) and pretreatment with RO 4-4602 on some variables of the sleep-wakefulness cycle during 8 h sessions starting 20 min after the last injection

Treatment (mg/kg)	*Wakefulness (min ± s.e. mean)*	*Slow wave sleep (min ± s.e. mean)*	*REM sleep (min ± s.e. mean)*
Control	137 ± 14	301 ± 13	42 ± 4
DOPS (80)	122 ± 25	318 ± 23	40 ± 4
DOPS (160)	121 ± 20	322 ± 20	37 ± 6
RO (50) + DOPS (80)	141 ± 18	306 ± 14	33 ± 4
RO (50) + DOPS (160)	113 ± 22	325 ± 16	42 ± 6

Differences in mean values were not significant.

80 mg/kg DOPS were much higher than control values during the third hour, but no significant changes of total wakefulness, SWS and REM time were observed during the 8 h sessions (Table 3; Figure 3). However, the latency for the first REM episode diminished significantly ($P < 0.01$) after 160 mg/kg DOPS in the RO 4-4602 pretreated animals (Figure 2)

Discussion

The administration of DOPA modified the spontaneous behaviour of the rats as well as their rate of cyclic alternation of sleep and wake phases When they received DOPA without previous inhibition of the peripheral decarboxylase, a decrease of ongoing motor activity and symptoms of increased autonomic activity became evident. Both have been related by Carlsson (1965) to catecholamines formed from DOPA at different peripheral sites. When the decarboxylation of DOPA at extracerebral sites was prevented, the behavioural modifications induced by the precursor had an opposite nature; that is, increased spontaneous motor activity together with stereotyped movements appeared. Butcher & Engel (1969) relate them to newly formed catecholamines (mainly dopamine) at different central sites.

During the first 20 min following the injection of DOPA with or without a peripheral decarboxylase inhibitor, a significant increase of slow wave activity was observed in the EEG. In spite of the electrocortical synchronization, the animals were aroused; that is, a dissociation between EEG and behaviour took place. Synchronization of the EEG followed by a prolonged waking state was also reported by Gaillard, Friedli & Tissot (1973) after intravenous infusion of DOPA in rabbits pretreated with a decarboxylase inhibitor. However, previous studies dealing with the actions of DOPA on the rat EEG, where the precursor was administered intraperitoneally, failed to show an initial synchronization, because the recordings were started 20-25 min after drug injection, when the EEG pattern had already shifted to one of fast and low voltage waves (Monnier, 1960; Thut & Rech, 1972).

There is evidence linking 5-hydroxytryptamine (5-HT) with the EEG components of SWS (Jouvet, 1969). Further, Ng, Chase, Colburn & Kopin (1970) found that DOPA markedly increased the efflux of 5-HT from rat brain slices, this action being contingent on the decarboxylation of DOPA to dopamine. In agreement with these *in vitro* results is the finding by Karobath, Diaz & Huttunen (1971) that intraperitoneal injection of DOPA decreases 5-HT levels in rat brain. Hence, it could be suggested that the initial synchronization after DOPA administration is related to the release of 5-HT.

The initial stage of increased EEG synchronization was later substituted by another of continuous EEG desynchronization, the duration of which was related to the dose of DOPA injected. After 80-160 mg/kg DOPA, waking time exceeded that of SWS during the first 60-120 minutes. When the peripheral decarboxylase was inhibited prior to DOPA injection, wakefulness was almost continuous during the first 120-180 min also surpassing SWS numerically during the first 180-240 minutes. Values for the entire 8 h sessions varied in a dose-related manner and inhibition of the peripheral decarboxylase, while increasing DOPA effects on the sleep-wakefulness cycle, failed to alter this relationship. REM followed a different course, which was characterized by an initial suppression lasting 60-240 min according to the treatment involved. Recovery was gradual with no further rebound.

The measurement of catecholamine levels in the central nervous system after DOPA injection by Butcher & Engel (1969), Everett & Borcherding (1970) and Benkert, Gluba & Matussek (1973) showed that the precursor produced a marked increase in brain dopamine, no change in NA and a decrease in 5-HT. When given to RO 4-4602 pretreated animals, the drug produced a further increase of central dopamine levels, while the NA content was not modified. However, Romero, Chalmers, Cottman, Lytle & Wurtman (1972) found that although total concentrations of NA are not modified after DOPA, the telencephalon, hypothalamus and cerebellum contain significantly more NA. It could be suggested, therefore, that both total dopamine and regional NA increases are related to the disruption of the sleep-wakefulness cycle found by us after DOPA administration.

DOPS did not significantly modify the sleep-wakefulness cycle apart from a decrease of the latency for the first REM episode after 160 mg/kg in the RO 4-4602 pretreated animals. In contrast to DOPA, blockade of peripheral decarboxylase did not increase DOPS effects on waking, SWS and REM. Havlicek (1967), using a different approach, found that intravenous infusion of 500 mg/kg DOPS was followed 2 h later by a very slight increase of REM together with an unusual kind of SWS, characterized by the fact that the animals were lying on their sides.

It is known that doses of DOPS in the range used by us produce a relatively slight increase in cerebral NA, while pretreatment with RO 4-4602 abolishes the increase (Bartholini, Constantinidis, Tissot & Pletscher, 1971; Benkert *et al.*, 1973).

These facts, most probably related to the low affinity of DOPS for the decarboxylase and to the inhibition by RO 4-4602 of DOPS entry into the brain (Holtz, 1959; Bartholini *et al.*, 1971), could tentatively explain the low amount of initial synchronization as well as the absence of significant changes of the sleep-wakefulness cycle variables after DOPS. Therefore, in order to determine the effects of a selective rise of NA on the sleep-wakefulness cycle, more suitable methods are needed

We are grateful to Dr P. Schellert of Hoffman-La Roche & Co. Ltd for generous supplies of DOPA and RO 4-4602. DL-threo-3,4-dihydroxyphenylserine was kindly provided by Dr A.A. Manian, Psychopharmacology Center, National Institute of Mental Health, Bethesda, Md., U.S.A.

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(Received August 26, 1974,
Revised December 3, 1974)

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STUDIES ON THE BEHAVIOURAL PHARMACOLOGY OF A CYCLIC ANALOGUE OF DOPAMINE FOLLOWING ITS INJECTION INTO THE BRAINS OF CONSCIOUS RATS

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1 The cyclic analogue of dopamine, 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (ADTN) was injected into the lateral ventricle or bilaterally into the nucleus accumbens or caudate nucleus of conscious rats and its effect on locomotor activity was investigated.

2 When given intraventricularly, ADTN produced some stereotyped responses which were followed by a strong and long lasting stimulation of locomotor activity. When administered bilaterally into the nucleus accumbens a similar stimulation of locomotor activity was observed. ADTN had no effect on locomotor activity when injected bilaterally into the caudate nucleus.

3 The ADTN-induced locomotor stimulation following its intraventricular injection was completely abolished by a low dose of pimozide (0.01 mg/kg, i.p.) or haloperidol (0.5 mg/kg, i.p.). Pimozide (1 mg/kg, i.p.) given 30 min before ADTN injected bilaterally into the nucleus accumbens completely blocked locomotor stimulation.

4 Unilateral injections of ADTN (5 μg) into the nucleus accumbens caused locomotor stimulation but no turning.

5 Bilateral injections into the nucleus accumbens of 2-amino-6,7-dimethoxy-1,2,3,4-tetrahydronaphthalene or 0.9% w/v NaCl solution had no effect on locomotor activity.

6 It is concluded that the central stimulant action of ADTN is due to an effect on the dopamine receptors in the nucleus accumbens.

Introduction

The functional role of dopamine in different aspects of animal behaviour has been reviewed by Hornykiewicz (1971) and Van Rossum (1970). It has been suggested that an increased locomotor activity can be produced by the stimulation of central dopamine receptors (Van Rossum & Hurkmans, 1964, Van Rossum, 1970). Dopamine may also have a major role in mediating turning behaviour in rats with unilateral lesions of the nigro-striatal dopamine tract (Ungerstedt, 1971).

The presence of dopamine in highly localized areas within the extrapyramidal system led to the suggestion that it may be involved in several physiological processes (Hornykiewicz, 1971), and in some extrapyramidal disorders (Papeschi, 1972; Klawans, 1973). The long dopamine-containing fibres arise from cell bodies in the substantia nigra (A9) or from cells caudal and dorsal to the substantia nigra (A8 and A10). These fibres terminate without crossing the midline in the striatum, the nucleus accumbens and the tuberculum olfactorium (see Dahlström & Fuxe, 1964). Microinjections of dopamine or ergometrine into the nucleus accumbens produce strong stimulation of locomotor activity in rats (Pijnenburg & Van Rossum, 1973; Pijnenburg, Woodruff & Van Rossum, 1973).

The tetralin derivative, 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (ADTN) contains the dopamine skeleton in a rigid molecule and might be expected to stimulate dopamine receptors (Woodruff, 1971). Recently it has been shown that ADTN causes strong and long-lasting stimulation of locomotor activity following its intraventricular administration into conscious mice and there is evidence that this action of ADTN is due to dopamine receptor activation (Woodruff, Elkhawad & Pinder, 1974b; Munday, Poat & Woodruff, 1974; Woodruff, Elkhawad, Crossman & Walker, 1974a).

In the experiments described in this paper we have investigated the site of the stimulant action of ADTN by injecting the compound into two areas of the rat brain where dopamine is highly

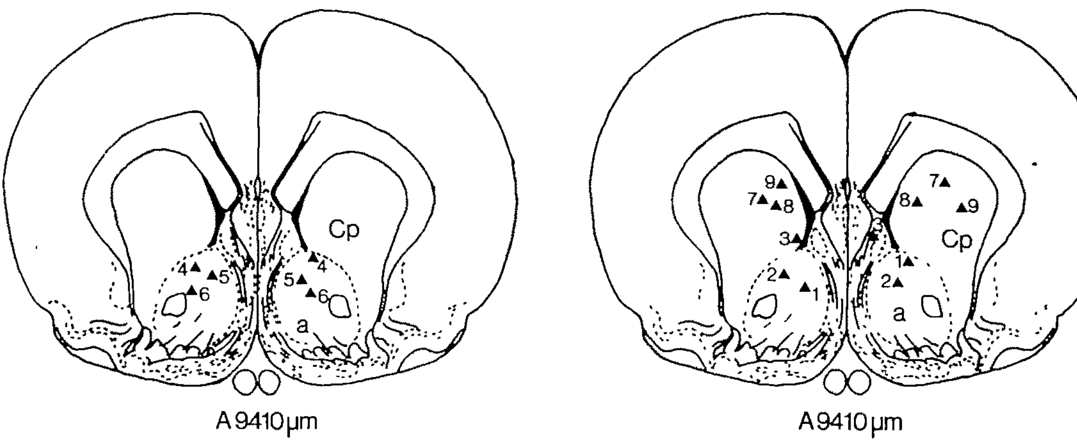

Figure 1 Frontal sections of the rat brain according to the atlas of Konig & Klippel (1963) in which the injection site is indicated by ▲. The numbers 1-6 refer to the rats with cannulae in the nucleus accumbens; the numbers 7-9 to rats with cannulae in the caudate nucleus Abbreviations: a: nucleus accumbens; Cp: caudate nucleus putamen.

localized. Since we have used rats for the experiments involving direct intracerebral injections it was first necessary to study the neuropharmacological actions of intraventricular injections of ADTN in conscious rats. The ability of some dopamine receptor antagonists, essentially neuroleptic drugs, to modify the behavioural actions of ADTN was also examined.

Methods

Intraventricular injections

Male rats (180-200 g) were lightly anaesthetized with a mixture of halothane, nitrous oxide and oxygen. Injections were made into the lateral ventricles by the method of Noble, Wurtman & Axelrod (1967). All drugs were dissolved in 0.9% w/v NaCl solution (saline) and injected unilaterally in a volume of 5 µl. The needle was allowed to stay in place for about one minute and then withdrawn. After recovery the animals were transferred to activity cages.

Intracerebral injections

Stainless steel cannulae were implanted bilaterally into the nucleus accumbens or caudate nucleus of male Wistar rats (200 g) under sodium pentobarbitone anaesthesia (50 mg/kg, i.p.). Coordinates for the caudate nucleus were A 9.4, L 2.0, H 1.0, (3 rats), the nucleus accumbens A 9.4, L 1.5, H—0.6, (6 rats) according to the atlas of König & Klippel (1963). The cannulae were fixed onto the skull with acrylic dental cement. Two stainless steel jeweller's screws were placed into the skull to firmly attach the cement. After recovery from the operations the animals were housed individually. The animals were allowed to recover for at least two weeks during which they were accustomed to handling and to the activity cages.

Injections were given by a 1 µl Hamilton syringe fitted within a 31 gauge needle. The tip of the needle was allowed to extend into the brain tissue 0.1 mm below the tip of the cannula. All drugs were dissolved in saline and injected in a volume of 0.5 µl. After the injection the animals were transferred to the activity cages and the behaviour observed for several hours. Motor activity of the rats was measured for a period of 24 h starting at 14 h 00 min each day. Animals had free access to food and water. The rats were allowed a period of at least 48 h before they were used again.

Measurement of locomotor activity

Locomotor activity was measured in conventional activity cages of the light beam type or in an Animex DSE activity meter. The former cages were equipped with three light sources and three photoelectric cells. The cages were circular, 36 cm in diameter and 25 cm high. The light sources and the photoelectric cells were equally distributed around the activity cage. The interruptions of the light beam were recorded on a Harvard cumulative recorder (Ralph Gerbrands). When using the Animex meter the animals were put singly in a perspex cage (38 x 26 x 25 cm) which was mounted on top of the activity meter. The apparatus was tuned in such a way that only large movements were recorded (sensitivity 10).

Histology

The animals were anaesthetized with sodium pentobarbitone (50 mg/kg, i.p.). They were perfused first with 30 ml of saline and then with 50 ml of Heidenhain's 'Susa' solution. The cannulae were carefully removed and the brains kept in Susa solution for a maximum period of 24 hours. Frontal sections (15 μm thick) were cut and stained with haematoxylin-eosin. The needle tracks were located and their tips marked on a frontal diagram of the rat brain (Figure 1).

Drugs

The following drugs were used: 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene hydrobromide (ADTN), 2-amino-6,7-dimethoxy-1,2,3,4-tetrahydronaphthalene hydrochloride. Both were dissolved in saline. Haloperidol and pimozide (Janssen Pharmaceutica) were both dissolved in 2% tartaric acid.

Results

Intraventricular injections

Following the unilateral intraventricular injection of ADTN a biphasic effect was observed. In the first 60 min there was backward walking, piloerection, stereotyped head movements and turning away from the site of injection. In some rats the circling was accompanied by a twisted body position. Jerky and uncoordinated movements were also observed. Those responses were followed by increased sensitivity to sound and by rearing. The rearing gradually increased in intensity. The rats showed strong locomotor stimulation which lasted for several hours. The rats often made quick rushes across the cage. The effect of intraventricular injection of different doses of ADTN is shown in Figure 2, which shows a dose-total count relationship. The counts represent the large movements of the animal which consisted of forward walking, rearing and running activity. Maximum stimulation was obtained after a dose of 200 μg of ADTN. The activity of animals injected intraventricularly with saline was not significantly different from untreated animals during a period of 20 h after injection. Intraventricular injections of 2-amino-6,7-dimethoxy-1,2,3,4-tetrahydronaphthalene had no stimulant effect on motor activity.

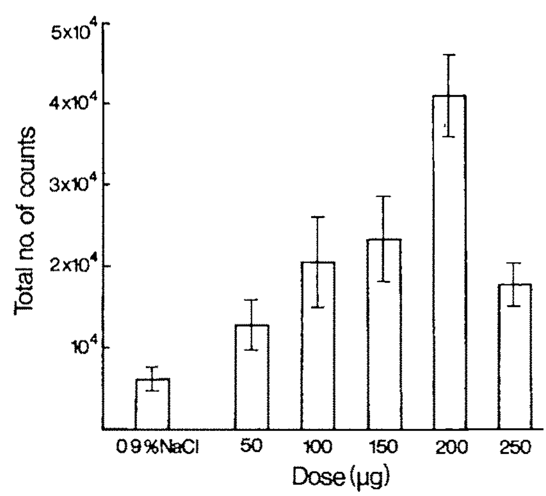

Figure 2 Dose-total counts relationship of the different doses of ADTN injected intraventricularly on one side. Activity was measured in the Animex activity meter. Maximum response was obtained at 200 μg. Each column is mean result from 6 rats. Vertical bars show s.e. mean.

The stimulation of locomotor activity produced by ADTN was not apparent until 1-2 h after the injection and then lasted between 12 and 18 h depending on the dose injected (Figure 3). The animals showed normal activity 24 h after the injection.

The ADTN-induced locomotor stimulation could be completely abolished by pimozide (0.01 mg/kg, i.p.) injected 30 min before ADTN (Figure 3). Haloperidol (0.5 mg/kg, i.p.) was similarly effective (Figure 3).

Injections into nucleus accumbens

Bilateral injections of ADTN into the nucleus accumbens, 5-25 μg on each side, produced initial sedation followed by strong central stimulation. During the first 15-60 min after injection shivering was observed. In some rats a flat posture was observed for a few minutes; these animals showed no signs of ataxia. The animals then became hyperactive with predominant rearing behaviour at first. Locomotor stimulation was strong and the animals moved in all directions in the cage. The running started suddenly and lasted for a very long period of time as shown in Figure 4a. During this period of enhanced activity the animals responded to handling, without undue signs of fear.

ADTN was effective in all 6 rats with cannulae in the nucleus accumbens. The tip of the cannula of rat number 3 (see Figure 1) was slightly above the nucleus accumbens, but the syringe needle track was detected inside the nucleus.

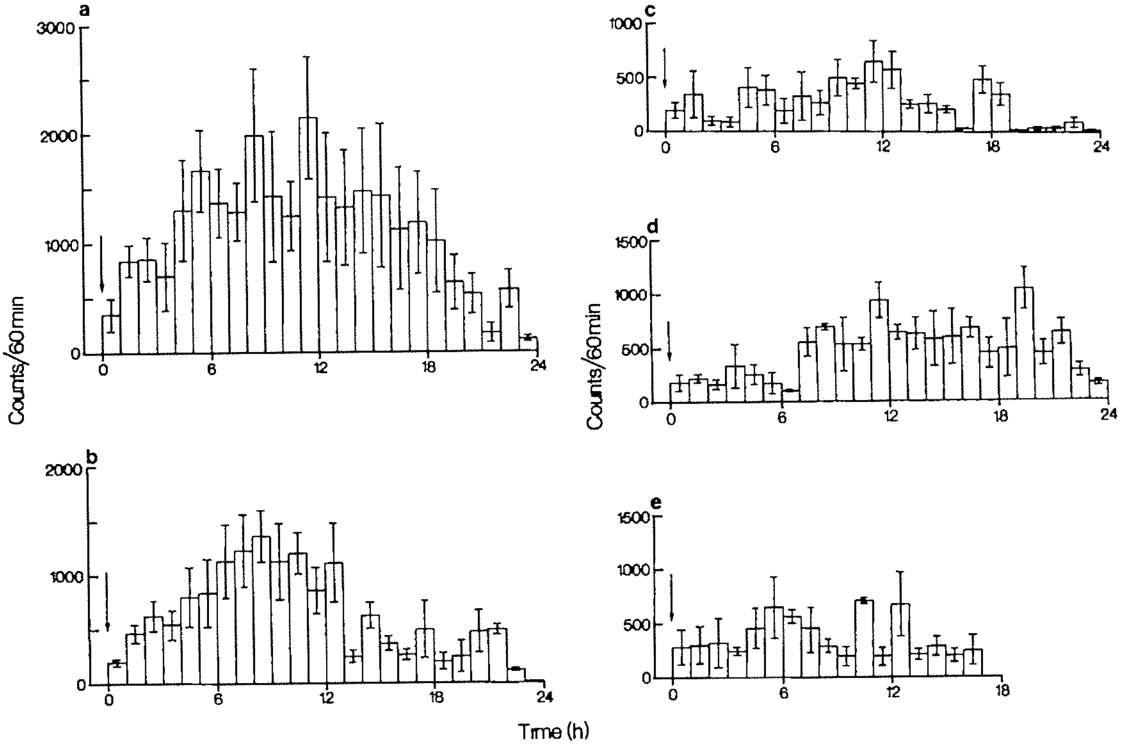

Figure 3 Mean motor activity of rats measured in the Animex activity meter. Vertical bars show standard errors of mean. Times of intraventricular injections are indicated by the arrows. (a) Motor activity (mean of 6 rats) after the intraventricular injection of 150 μg of ADTN unilaterally. (b) Motor activity (mean of 3 rats) following the injection of ADTN 150 μg given unilaterally into the lateral ventricle 30 min after haloperidol (0.1 mg/kg, i.p.). The effect of ADTN was attenuated. (c) Motor activity (mean of 3 rats) following the injection of 5 μl saline unilaterally into the lateral ventricle. (d) Motor activity (mean of 4 rats) following the injection of ADTN 150 μg given unilaterally into the lateral ventricle 30 min after pretreatment with pimozide (0.01 mg/kg, i.p.). The effect of ADTN was abolished. (e) Motor activity (mean of 3 rats) following the injection of ADTN 150 μg 30 min after pretreatment with haloperidol (0.5 mg/kg, i.p.).

Enhanced locomotor activity was also produced by lower doses of ADTN (0.5, 2 or 5 μg on each side). All 6 rats responded to the bilateral injection of 5 μg of ADTN (Figure 5a). The onset of action was slower and the duration shorter than with the higher dose. After 2 μg of ADTN, two out of three rats tested showed increased activity, and one rat responded to the lower dose of 0.5 μg.

Unilateral injections of ADTN (5 μg) into the nucleus accumbens also caused enhanced locomotor activity but there was no turning. The onset of action was delayed but the duration of action seemed to be unchanged compared with that after bilateral injections (Figure 5).

Pimozide (1 mg/kg, i.p.) injected 30 min before ADTN completely abolished the locomotor stimulation produced by the bilateral injection of 5 μg ADTN (Figure 5). The effects of the same dose of ADTN were greatly attenuated by pimozide (0.4 mg/kg) (Figure 5). With lower doses of pimozide (0.1 or 0.2 mg/kg) the effect of ADTN was not blocked although the onset of action was delayed.

The injection of 0.5 μl of saline or 25 μg 2-amino-6,7-dimethoxy-1,2,3,4-tetrahydronaphthalene bilaterally into the nucleus accumbens had no effect on locomotor activity as shown in Figure 4.

Injections into caudate nucleus

When ADTN (25 μg) was injected bilaterally into the caudate nucleus it had no effect on locomotor activity (Figure 4), although some stereotyped sniffing and licking were observed. Piloerection and flat posture were shown by some rats.

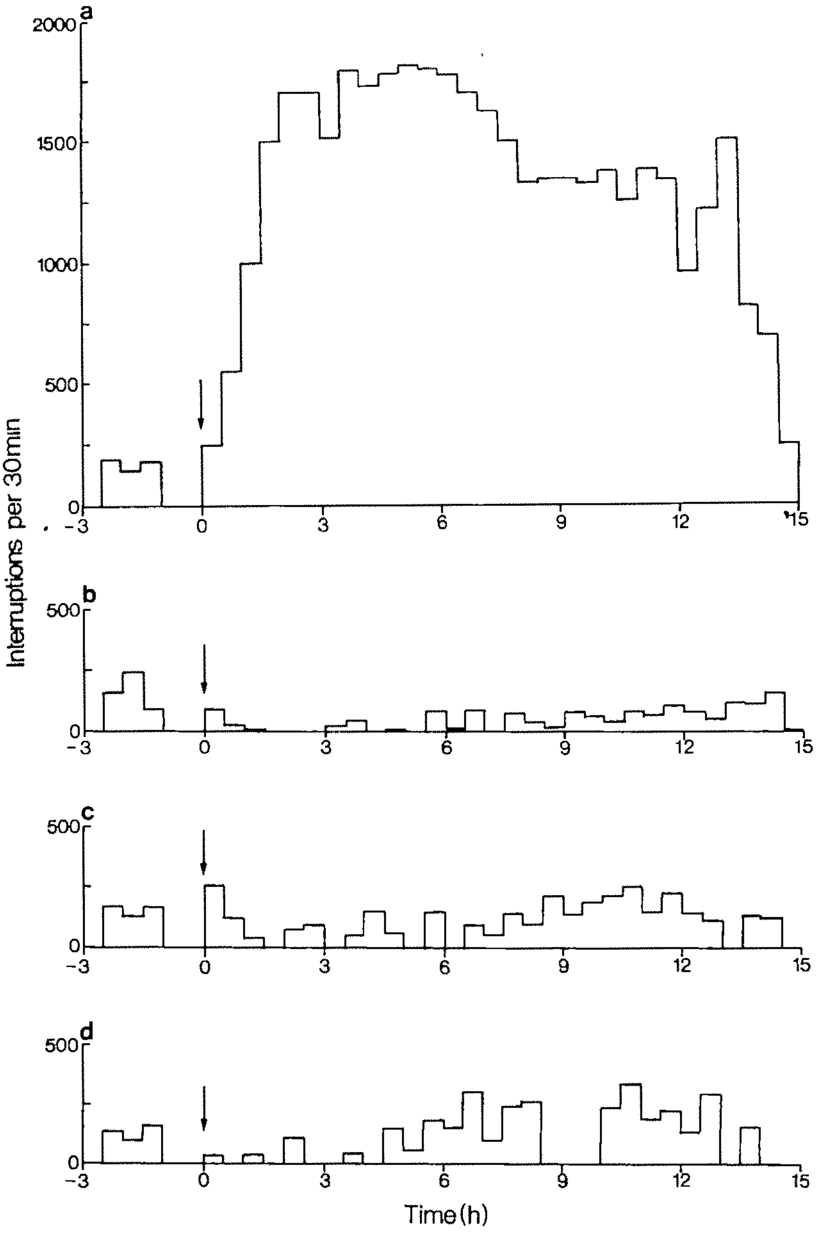

Figure 4 Motor activity of rats as measured in the light beam activity cages. Times of intracerebral injections are indicated by the arrow. (a) Motor activity of rat No. 1 following the injection of 25 μg of ADTN bilaterally on each side, into the nucleus accumbens. (b) Motor activity of the same rat after injection of 2-amino-6, 7-dimethoxy-1,2,3,4-tetrahydronaphthalene, 25 μg bilaterally on each side into the nucleus accumbens. (c) As a control the motor activity of the same rat is shown following the injection of 0.5 μl saline bilaterally into the nucleus accumbens. (d) Motor activity of rat No. 8 after the injection of ADTN 25 μg bilaterally into the caudate nucleus.

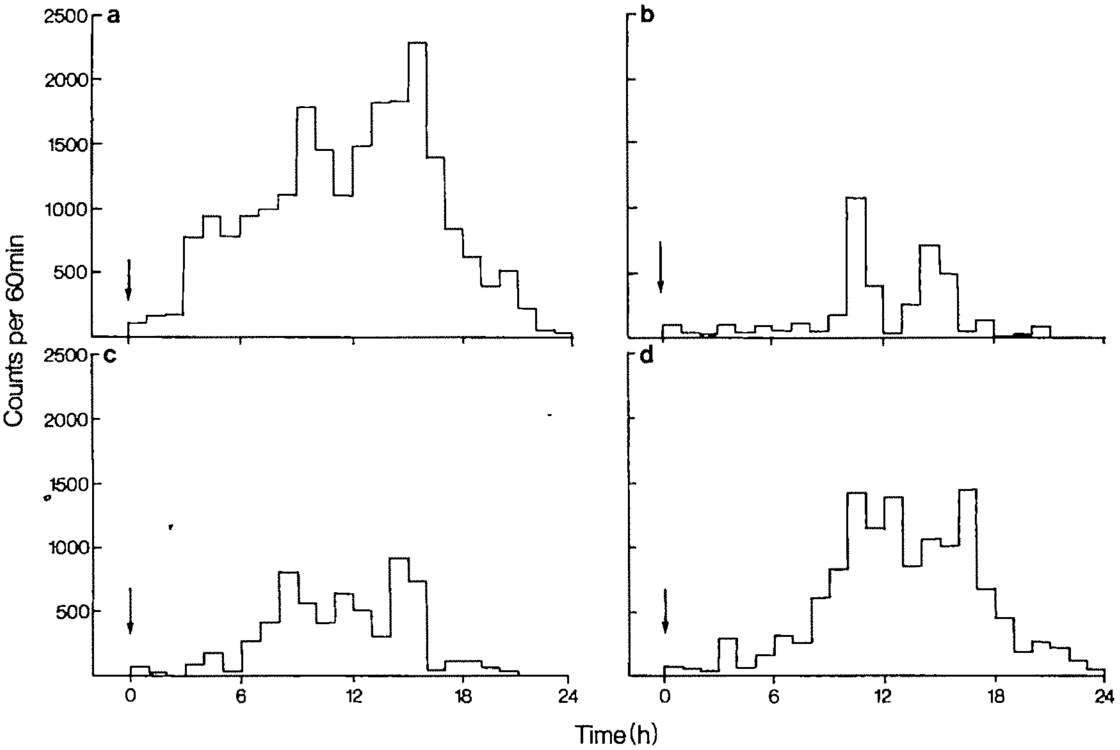

Figure 5 Motor activity of rats measured in the Animex activity meter. Times of intracerebral injections indicated by the arrows. (a) Motor activity of rat No. 6 after an injection of ADTN 5 μg bilaterally into the nucleus accumbens. (b) Motor activity of the same rat after pretreatment with pimozide (1 mg/kg, i.p.) 30 min before the injection of ADTN 5 μg bilaterally into the nucleus accumbens. Activity is completely inhibited. (c) Motor activity of the same rat after pretreatment with pimozide (0.4 mg/kg, i.p.) 30 min before the injection of ADTN 5 μg bilaterally into the nucleus accumbens. The effect is greatly attenuated. (d) Motor activity of rat No. 4 following the injection of ADTN 5 μg unilaterally (left side) into the nucleus accumbens.

Discussion

There is a large body of evidence to suggest that many of the actions of dopamine, both peripheral and central, are mediated by specific dopamine receptors (Hornykiewicz, 1971; Woodruff, 1971; Goldberg, 1972). The discovery by Ehringer & Hornykiewicz (1960) that there is a deficiency of dopamine in the basal ganglia of parkinsonian patients led to the introduction of L-DOPA as a drug for its treatment (Cotzias, Van Woert & Schiffer, 1967). Drugs which stimulate dopamine receptors could be of great value in the therapy of parkinsonism and also as a research tool in the study of the physiological significance of dopamine.

In our present study we have shown that ADTN causes a strong and long-lasting stimulation of locomotor activity in rats. The effect is probably mediated by an action on dopamine receptors. This is supported by the fact that low doses of haloperidol and pimozide antagonized the effect of ADTN. We have shown in a previous experiment that the effect of ADTN on locomotor activity in mice is not affected by the α-adrenoceptor blocking agent phenoxybenzamine (unpublished observation) nor by pretreatment with the inhibitor of dopamine synthesis, α-methyl-*p*-tyrosine (Woodruff *et al.*, 1974b). There is good evidence from other experiments that ADTN stimulates mammalian dopamine receptors. Thus, ADTN has been shown to stimulate the production of cyclic adenosine 3′,5′-monophosphate in the rat striatum (Munday *et al.*, 1974; Miller, Horn, Iversen & Pinder, 1974). In addition it has been shown that ADTN induces turning behaviour in rats with unilateral lesions of the nigro-striatal tract which is consistent with a direct action of this compound on dopamine reeptors, an effect which was also reported by Woodruff *et al.* (1974a). Furthermore, ADTN was shown to exhibit inhibitory actions on dopamine-sensitive neurones in the caudate nucleus when applied iontophoretically (Woodruff *et al.*, 1974a). In the peripheral vascular system it has been shown that ADTN had a vasodilator activity on the renal arteries of the dog similar to that of dopamine (Goldberg & Crumly, personal communication), whereas it had no effect on the femoral arteries. ADTN also has a dopamine-like action on the guinea-pig blood pressure (Woodruff *et al.*, 1974b).

The dimethyl ether of ADTN, 2-amino-6,7-dimethoxy-1,2,3,4-tetrahydronaphthalene has little or no effect on striatal dopamine receptors (unpublished observation). In the present study we have shown that this compound is similarly inactive behaviourally.

Thus our results support the concept that dopamine receptors are involved in locomotor stimulation of drugs. The evidence that the actions of ADTN are not affected by the tyrosine hydroxylase inhibitor, α-methyl-*p*-tyrosine, (Woodruff *et al.*, 1974b), and its effect on turning behaviour strongly supports the concept that ADTN acts by a direct action on dopamine receptors.

A remarkable feature of the actions of ADTN has been the prolonged effects in mice and rats, following intraventricular injections and injections into the nucleus accumbens. The long lasting effects of ADTN might be due to its resistance to enzymatic action in the brain, or its removal from the receptor vicinity at a very slow rate. However, recently it has been shown (Horn, 1974) that ADTN is a potent inhibitor of dopamine uptake into rat striatal homogenates; these results could mean that ADTN is itself taken up presynaptically. Other possible explanations for the long duration of action of ADTN, such as the formation of a stable complex with the dopamine receptor, cannot be ruled out.

Evidence presented by Rolls (1971) suggests that the nucleus accumbens may be a site of self stimulation. Our experiments suggest that the nucleus accumbens may be involved in the site of action of ADTN. This is supported by the finding that ADTN produces strong stimulation of locomotor activity following its injection into the nucleus accumbens but is inactive when injected into the caudate nucleus. The nucleus accumbens contains high dopamine levels and a dopamine-sensitive adenyl cyclase (Horn, Cuello & Miller, 1974). Thus the nucleus accumbens may be involved in the mediation of locomotor stimulant activity of drugs. Recently, it has been shown that injections of dopamine or the dopamine receptor stimulant, ergometrine, into the nucleus accumbens produce stimulation of locomotor activity in rats (Pijnenburg & Van Rossum, 1973; Pijnenburg *et al.*, 1973).

Intraperitoneal injections of ADTN produce no obvious behavioural changes, probably because the molecule does not readily penetrate the blood-brain barrier. The study of more lipid soluble analogues of ADTN might produce active compounds that are capable of passing from the blood into the brain.

We are very grateful to Dr John Mitchell for help with the histology. Our thanks are due to Dr Paul Janssen for gifts of haloperidol and pimozide and to Dr Roger Pinder for ADTN. The cannulae used in these experiments were kindly donated by Professor J.M. Van Rossum. A.O.E. was supported by a research scholarship from the University of Khartoum.

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(Received October 29, 1974.
Revised January 9, 1975)

Br J. Pharmac. (1975), 54, 115-121

THE INTERACTION OF NEUROLEPTIC AND MUSCARINIC AGENTS WITH CENTRAL DOPAMINERGIC SYSTEMS

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1 The effect of muscarinic and neuroleptic agents on the turning behaviour induced by methamphetamine and apomorphine in rats with unilateral lesions of the substantia nigra induced by 6-hydroxydopamine has been examined.

2 Turning towards the side of the lesion induced by (+)-methamphetamine (5 mg/kg) was inhibited by α-flupenthixol (1 mg/kg) and α-clopenthixol (8 mg/kg) but not by high doses of their β-isomers.

3 Turning was inhibited by chlorpromazine (4 mg/kg) and pimozide (0.2 mg/kg). Thioridazine and clozapine (16 mg/kg) were ineffective. Turning in the same direction produced by scopolamine (10 mg/kg) was also inhibited by α-flupenthixol (1 mg/kg) and pimozide (0.25 mg/kg).

4 Turning produced by methamphetamine (5 mg/kg) was inhibited by oxotremorine (0.75 mg/kg) even in the presence of methylatropine (5 mg/kg).

5 Turning away from the side of the lesion induced by apomorphine (0.1 mg/kg) was inhibited by oxotremorine (0.75 mg/kg) but not by thioridazine or clozapine (16 mg/kg).

6 These results are discussed with regard to the mode of action of neuroleptic drugs in producing anti-psychotic effects and drug-induced Parkinsonism.

Introduction

Apart from their therapeutic antipsychotic action neuroleptic drugs may produce unwanted side-effects. One commonly observed side-effect is the occurrence of drug-induced parkinsonian symptoms (Klawans, 1973). Both the anti-psychotic action and the extrapyramidal parkinsonian side-effects of the neuroleptic drugs generally correlate well with their potency in blocking dopamine receptors as revealed by *in vitro* (Clement-Cormier, Kebabian, Petzold & Greengard, 1974; Horn, Cuello & Miller, 1974; Miller, Horn & Iversen, 1974) and *in vivo* (Andén, Butcher, Corrodi, Fuxe & Ungerstedt, 1970) models. However, it has been reported that some neuroleptics, notably thioridazine and clozapine, produce only minimal parkinsonian symptoms (Cole & Clyde, 1961; Burki, Ruch, Asper, Baggiolini & Stille, 1973) although they are potent blockers of dopamine effects *in vitro* (Miller *et al.*, 1974).

Since drug-induced extrapyramidal effects can be alleviated by antimuscarinic drugs (Shintani & Yamamura, 1973) it is possible that the recently demonstrated antimuscarinic action of clozapine and thioridazine (Miller & Hiley, 1974; Snyder, Greenberg & Yamamura, 1974) might account for their production of only minimal parkinsonian side-effects. Parkinsonism is related to deficient striatal dopaminergic function (Hornykiewicz, 1973) and drug-induced Parkinsonism may therefore result from blockade of striatal dopamine receptors. A possible basis for the alleviation of parkinsonian symptoms by antimuscarinic agents is the observed antagonistic effects of muscarinic agents and drugs acting on dopamine receptors in the striatum (Costall, Naylor & Olley, 1972).

In the present investigation we have used the rotation of rats with unilateral degeneration of the nigrostriatal pathway (Ungerstedt & Arbuthnott, 1970; Ungerstedt, 1971) as a quantitative *in vivo* model of striatal dopaminergic activity to study the action of cholinomimetic agents and neuroleptics on striatal dopaminergic mechanisms.

Methods

Male albino Sprague-Dawley rats (250-300 g at the time of surgery) were given food and water *ad libitum* during the period of use.

Lesions

Animals were anaesthetized with Equithesin (Jensen-Salsbery Labs.), 2.5 ml/kg. They were then positioned in a stereotaxic apparatus and injected unilaterally with 6-hydroxydopamine through a 30 gauge stainless steel cannula aimed at the substantia nigra. The co-ordinates of the cannula tips were 2.8, 2.0, 8.0 according to the atlas of Pellegrino & Cushman (1967). 6-Hydroxydopamine hydrobromide was freshly dissolved (2 mg of base per ml) in cold 0.9% w/v NaCl solution (saline) containing 1 mg/ml of ascorbic acid; 4 µl of this solution was injected through the cannula at a rate of 1 µl/minute.

Turning behaviour

Drugs were made up in saline. The doses were calculated as the forms given below. Pimozide was first dissolved in a drop of glacial acetic acid and then diluted. Clozapine was dissolved with an equal weight of tartaric acid. Injection of vehicle solutions of these drugs into rats did not affect turning behaviour. All drugs were given intraperitoneally, except for apomorphine which was given subcutaneously. Fourteen days after the injection of 6-hydroxydopamine, the animals were screened for ability to turn with apomorphine (0.5 mg/kg) and (+)-methamphetamine (5 mg/kg). Only animals which responded with at least ten turns per minute were retained. Animals used in experiments with methamphetamine-induced turning were screened at least 10 days after operation, and those on apomorphine turning after at least three weeks. After initial selection, the animals were separated into groups of 4 or 5 animals and used in experiments with various drugs, at intervals of at least one week. The control responses of the various groups to methamphetamine and apomorphine during the period of the experiments did not change significantly. The experimental procedure was as follows. Animals were injected with the test drugs or vehicles 3 h before the experiments with the exception of oxotremorine which was given 5 min after the amphetamine or apomorphine. At the time of the experiment animals were injected with amphetamine or apomorphine and then placed in individual cages. All animals in a group received the same drug treatments. At 30 min ,intervals animals were placed in rotometer bowls for 5 minutes. The number of turns per min was measured on the fifth min by direct observation. The rotometers consisted of white perspex translucent bowls supported on metal rings. The bowls measured 30 cm in diameter and 26 cm in height.

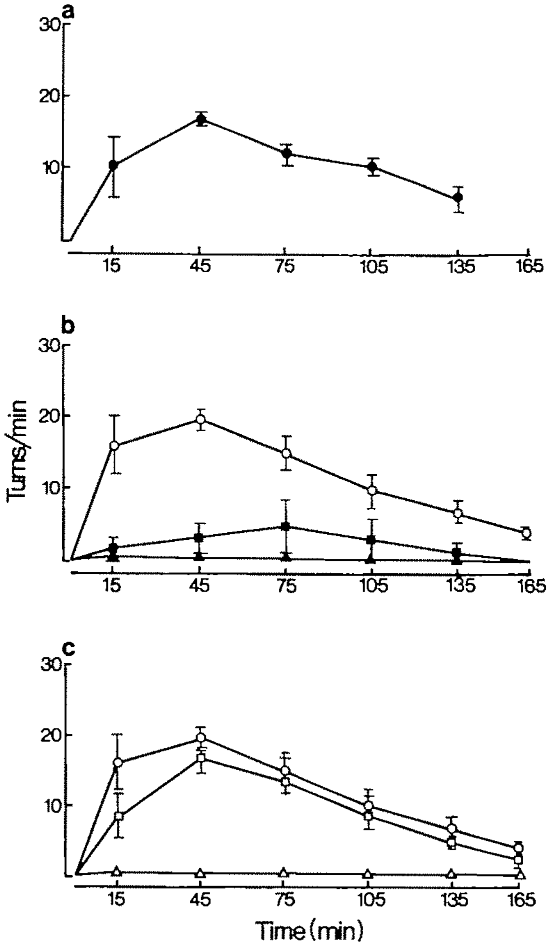

Figure 1 Effect of different isomers of thioxanthene neuroleptics on turning behaviour induced by methamphetamine (5 mg/kg). (a) Turning after 10 mg/kg β-flupenthixol (●). (b) Turning after 0.2 mg/kg (■) or 1.0 mg/kg (▲) α-flupenthixol and saline control (○). (c) Turning after 8 mg/kg α- (△) or β-clopenthixol (□) and saline control (○). Results are means from four rats Vertical bars show s.e. mean.

Drugs

The following drugs were used: (+)-methamphetamine hydrochloride, 6-hydroxydopamine

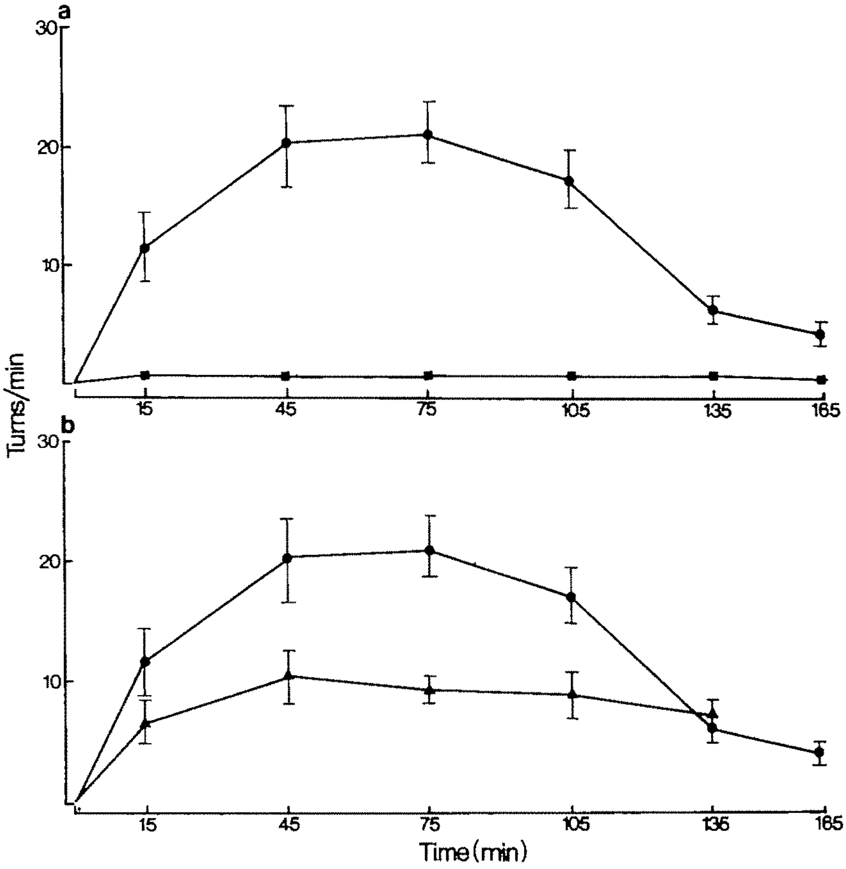

Figure 2 Effect of pimozide and chlorpromazine on methamphetamine- (5 mg/kg) induced turning behaviour. (a) Turning after 0.25 mg/kg pimozide (■) or saline (●). (b) Turning after 4 mg/kg chlorpromazine (▲) or saline (●). Results are means from four rats. Vertical bars show s.e. mean.

hydrobromide, (Sigma); thioridazine hydrochloride (Sandoz), clozapine (base) (Sandoz/Wander); scopolamine hydrochloride (BDH); apomorphine hydrochloride (MacFarlane Smith); oxotremorine sesquifumarate (Aldrich); pimozide (base) (Janssen); α- and β-flupenthixol and α- and β-clopenthixol dihydrochloride (Lundbeck).

Results

Blockade of methamphetamine-induced turning by cis- and trans-thioxanthene isomers

In accordance with previous reports (Ungerstedt & Arbuthnott, 1970) intraperitoneal injection of methamphetamine in lesioned animals produced turning towards the side of the lesion in a dose-dependent fashion. The thioxanthene neuroleptics flupenthixol and clopenthixol exhibit geometric isomerism, existing as *cis* (α) or *trans* (β) isomers. Previously we have shown that only the α-isomers are effective antagonists of the dopamine-stimulated adenylate cyclase in homogenates of rat striatum. Figure 1 shows the effects of the different isomers on turning produced by 5 mg/kg of methamphetamine. For both neuroleptics the β-isomers were ineffective whereas the α-isomers were effective in blocking such turning. α-Flupenthixol was more potent than α-clopenthixol. This again parallels the activity of the drugs on the dopamine-sensitive adenylate cyclase.

Effects of other neuroleptics

Pimozide (Figure 2) completely abolished turning behaviour at low doses. At 4 mg/kg chlorpromazine partially inhibited turning (Figure 2). This result is similar to that reported by Crow & Gillbe (1973). These authors also showed that

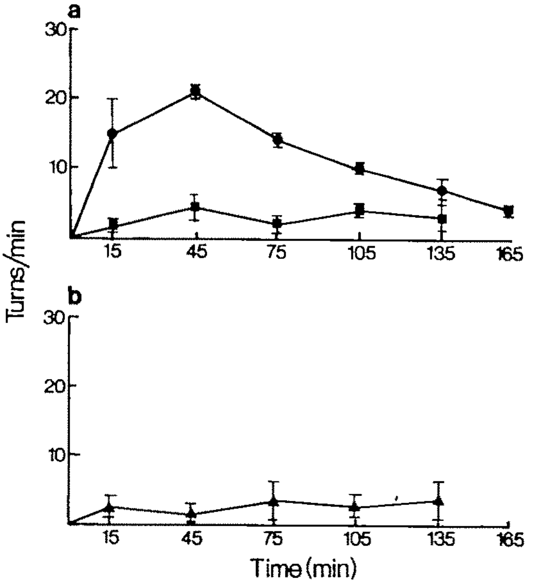

Figure 3 Effect of oxotremorine on turning behaviour induced by methamphetamine (5 mg/kg). (a) Turning after 0.75 mg/kg oxotremorine (■) given 5 min after the amphetamine and saline control (●). (b) Effect of 0.75 mg/kg oxotremorine and 5 mg/kg methylatropine (▲) given 5 min after the amphetamine. Results are means from four rats. Vertical bars show s.e. mean.

higher doses of chlorpromazine completely blocked turning. In contrast the two neuroleptics clozapine and thioridazine had no effect on turning behaviour even at very high doses (16 mg/kg). Similar observations with thioridazine at lower doses have been reported previously (Crow & Gillbe, 1973).

Effect of muscarinic agents

When 0.75 mg/kg oxotremorine was administered just after methamphetamine (5 mg/kg) the resulting turning behaviour was considerably reduced. In order to see whether this blockade of turning was due to central or peripheral effects of oxotremorine, some animals were also given 5 mg/kg of methylatropine, to block peripheral muscarinic receptors. In these animals oxotremorine (0.75 mg/kg) still produced a considerable decrease in methamphetamine-induced turning behaviour (Figure 3). In confirmation of previous reports (Ungerstedt, Avemo, Avemo, Ljungberg & Ranje, 1973), the antimuscarinic drug scopolamine (10 mg/kg) produced turning behaviour in the same direction as methamphetamine. However, scopolamine was considerably less effective than methamphetamine. Scopolamine-induced turning could be inhibited by α-flupenthixol and pimozide (Figure 4).

Effect of drugs on apomorphine-induced turning

When animals were given apomorphine, a directly acting dopamine receptor agonist, the animals turned away from the side of the lesion in a dose-dependent fashion. This phenomenon has been attributed to the action of apomorphine on supersensitive receptors on the lesioned side (Ungerstedt, 1971). Turning could be elicited by very low doses of apomorphine, down to 0.01 mg/kg. With doses of 0.05 mg/kg or higher the maximal number of turns per minute did not increase but the duration of turning did. It was found that oxotremorine (0.75 mg/kg) reduced apomorphine-induced turning (Figure 5). It was

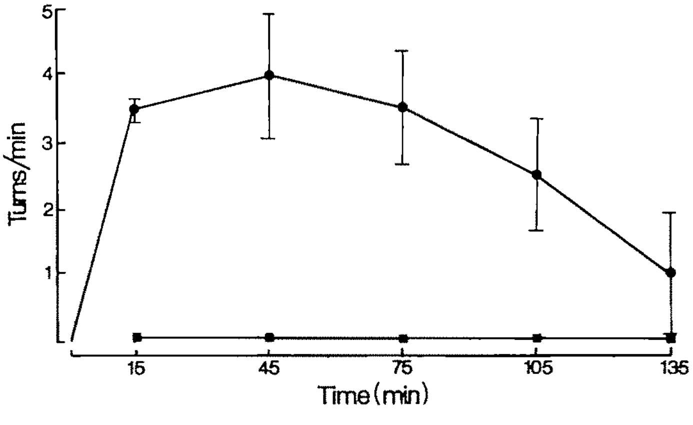

Figure 4 Turning towards the side of the lesion produced by scopolamine (10 mg/kg) (●). The turning was blocked by pimozide (0.25 mg/kg) (■) or α-flupenthixol (1.0 mg/kg) (■). Results are means from four rats. Vertical bars show s.e. mean.

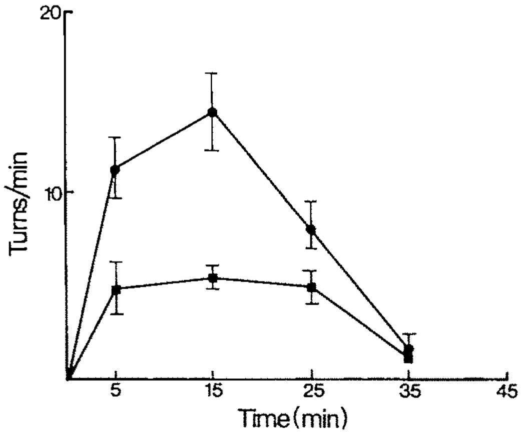

Figure 5 Effect of oxotremorine 0.75 mg/kg (■) or saline (●) given 5 min after apomorphine on turning behaviour produced by 0.1 mg/kg apomorphine. Results are means from four rats. Vertical bars show s.e. mean.

also found that clozapine and thioridazine (16 mg/kg) were ineffective in inhibiting apomorphine-induced turning (Figure 6).

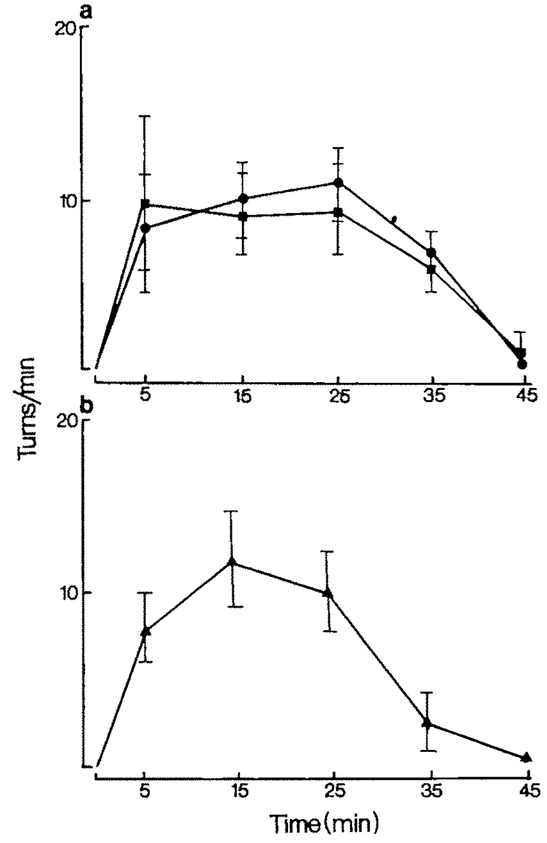

Figure 6 Effect of clozapine and thioridazine on turning induced by apomorphine (0.1 mg/kg). (a) Effect of thioridazine (16 mg/kg) (■) and saline control (●). (b) Effect of clozapine (16 mg/kg) (▲). Results are means from four rats. Vertical bars show s.e. mean.

Discussion

In the present investigation we have confirmed that some neuroleptic drugs are able to block the methamphetamine-induced rotation of rats with unilateral degeneration of the nigrostriatal pathway. The potency of chlorpromazine and the α- and β-isomers of flupenthixol and clopenthixol as amphetamine antagonists in this test correlated well with the dopamine blocking potencies of these drugs previously measured in an *in vitro* biochemical system (Miller *et al*, 1974). However, the neuroleptics thioridazine and clozapine did not block the turning provoked by methamphetamine or apomorphine even in high doses. On the basis of their original observation that thioridazine did not block methamphetamine-induced turning Crow & Gillbe (1973) suggested that thioridazine does not block dopamine receptors. However, both clozapine and thioridazine are potent blockers of the stimulation by dopamine of adenylate cyclase in homogenates of the rat striatum (Miller *et al.*, 1974), an *in vitro* system which has proved a useful model of the dopamine receptor.

An alternative explanation of the failure of thioridazine and also of clozapine to block methamphetamine-induced rotation is that their antimuscarinic action (Miller & Hiley, 1974; Snyder *et al.*, 1974) may alleviate the anti-dopaminergic effects of this drug in the striatum. We have demonstrated that central cholinergic stimulation produced by oxotremorine can block methamphetamine-induced turning and conversely that the antimuscarinic agent scopolamine produces turning in the same direction as methamphetamine.

The neuroanatomical basis of these cholinergic-dopaminergic interactions could involve both the substantia nigra and the striatum. Local application of cholinomimetic agents to the substantia nigra decreases the turnover of dopamine in nigrostriatal dopaminergic neurones, while antimuscarinic drugs have the opposite effect (Javoy, Agid, Bouvet & Glowinski, 1974), suggesting that cholinergic synapses are involved in an inhibitory effect on nigrostriatal neurones. The turning produced by the anti-acetylcholine agent, scopolamine, may involve activity in the intact nigrostriatal dopaminergic neurones, since this turning could be blocked by dopamine receptor blocking drugs such as pimozide and α-flupenthixol.

A further site of cholinergic-dopaminergic interaction is probably the striatum itself. In the present investigation we have observed that the turning produced by the dopamine agonist apomorphine is inhibited by the cholinomimetic agent oxotremorine. Since apomorphine-induced turning is thought to result from the direct stimulation of the supersensitive denervated striatal dopamine receptors (Ungerstedt, 1971), the effect of oxotremorine on apomorphine-induced turning does not depend on an action on nigrostriatal dopaminergic neurones One mechanism which may be important in the effect of oxotremorine on apomorphine-induced turning is an increase of striatal cholinergic activity. This would tend to antagonize dopaminergic activity since there is evidence that dopamine inhibits cholinergic interneurones in the striatum. For example, blockade of dopaminergic transmission by neuroleptics increases the release of acetylcholine from the caudate nucleus (Stadler, Lloyd, Gadea-Ciria & Bartholini, 1973) and reduces striatal levels of acetylcholine (Sethy & Van Woert, 1974; Agid, Guyenet, Javoy, Beaujouian & Glowinski, 1974). Treatment with dopamine agonists such as apomorphine and L-DOPA increases striatal levels of acetylcholine (Sethy & Van Woert, 1974).

In conclusion, using the rotation model we have demonstrated an inhibitory interaction between cholinergic activity and dopaminergic activity in the extrapyramidal system. Evidence has been discussed that this interaction can occur both in the substantia nigra and in the striatum itself. These interactions may explain why neuroleptic drugs such as thioridazine and clozapine which possess both antidopaminergic and antimuscarinic actions do not block the turning behaviour produced by dopamine release (methamphetamine) or by direct stimulation of dopamine receptors (apomorphine). The antimuscarinic actions of these drugs may also be responsible at least in part, for their lack of production of extrapyramidal symptoms when used clinically. Their therapeutic antipsychotic activity may therefore result from blockade of dopamine receptors other than those in the striatum.

R.M. is an M.R.C. Scholar. P.H.K. acknowledges the support of an ICI Fellowship. We are indebted to Lundbeck Ltd. for supplies of thioxanthene isomers, to Sandoz/Wander for clozapine and thioridazine and to Janssen Pharmaceutica for pimozide. We also acknowledge help and advice from Drs L.L. and S.D. Iversen during the course of this study.

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(Received October 16, 1974.
Revised December 23, 1974)

Br. J. Pharmac. (1975), 54, 123-124

HYPOTHERMIC ACTION OF BROMOCRIPTINE

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The body temperature of rats has been studied in a cold environment. In this setting, bromocriptine induced hypothermia. This fall in core temperature was inhibited by pimozide. It was concluded that bromocriptine has a dopamine-like action in areas of the brain concerned with the control of body temperature, as has previously been shown for apomorphine and amphetamine.

Introduction Drugs such as amphetamine and apomorphine reduce the core temperature of rats placed in a cold (4-15°C) ambient temperature. This hypothermic action is produced by dopaminergic mechanisms in the central nervous system (Kruk, 1972; Yehuda & Wurtman, 1972). It has recently been suggested that bromocriptine (2-brom-α-ergocryptine; CB 154 Sandoz) stimulates dopaminergic neurones (Corrodi, Fuxe, Hokfelt, Lidbrink & Ungerstedt, 1973). We have investigated the effect of bromocriptine on the colonic temperature of rats in an attempt to establish further evidence of the dopaminergic activity of this compound in the central nervous system.

Methods Drugs were administered intraperitoneally to male Wistar rats weighing 150-200 g. In each series of observations 5-13 animals were placed in individual perspex cages (20 x 10 x 10 cm) in a refrigerated room 1 h before starting observations. The intracolonic temperature was recorded every 10 min with a thermistor (Yellow Spring International Co., Yellow Springs, Ohio, U.S.A.) inserted 5-6 cm into the rectum. Ambient temperature in the cold room was maintained at 5-6°C. Observations were made following administration of bromocriptine 5-20 mg/kg. Bromocriptine was dissolved in a minimum volume of ethanol and appropriate dilutions in 0.9% w/v NaCl solution (saline) were made to allow the dose to be administered in a total volume of 0.7-1.0 ml. The effect of the ethanol saline vehicle alone was observed. Temperatures were also recorded following blockade of dopamine receptors with pimozide, which was dissolved in a minimum volume of glacial acetic acid, and then diluted.

Results The colonic temperature began to fall 10 min after intraperitoneal injection of bromocriptine 5-20 mg/kg. This hypothermic response was sustained for the full 1.5 h over which the

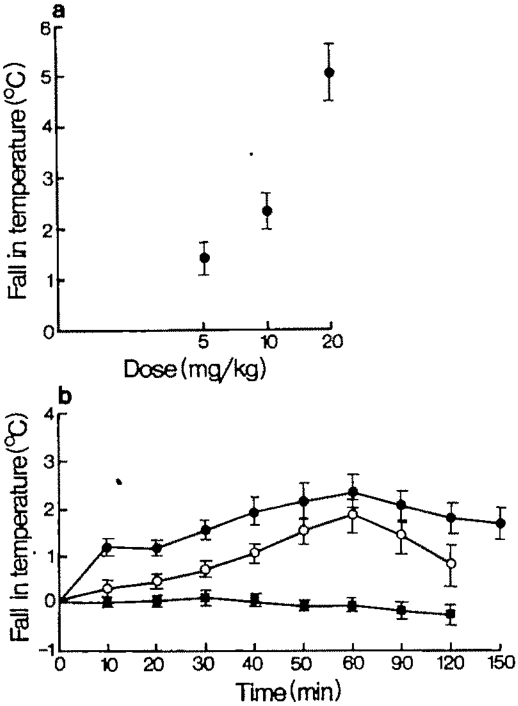

Figure 1 (a) Dose-response relationship for the maximum fall in colonic temperature induced by bromocriptine. The vertical bars show s.e. mean. (b) Inhibition of the hypothermic action of bromocriptine by pimozide. (●) Bromocriptine 10 mg/kg; (○) bromocriptine 10 mg/kg, 1 h after pretreatment with intraperitoneal pimozide, 1 mg/kg; (■) vehicle without drugs. The vertical bars show s.e. mean.

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temperature was recorded. The hypothermia was dose-dependent, 20 mg/kg of bromocriptine producing a fall in temperature of 5° C (Figure 1a).

The dopamine receptor blocking drug pimozide (Andén, Butcher, Corrodi, Fuxe & Ungerstedt, 1970) was administered 1 h before bromocriptine in order to establish whether the fall in temperature could be modified by inhibition of dopamine receptors. Pimozide reduced the hypothermic action of bromocriptine (Figure 1b).

Administration of the ethanol saline vehicle intraperitoneally caused short-lasting, 10-15 min sedation but had no effect on colonic temperature (Figure 1b).

Discussion Bromocriptine decreases prolactin secretion (Del Pozo, Brun, Varga & Friesen, 1972) and has been employed to decrease lactation (Varga, Lutterbeck, Pryor, Wenner & Erb, 1972). The mechanism of prolactin suppression is thought to be a dopaminergic effect on the hypothalamic pathways concerned with secretion of prolactin inhibitory factor into the portal circulation of the anterior pituitary. Bromocriptine has been found to induce rotational behaviour in rats with unilateral nigrostriatal lesions induced by 6-hydroxydopamine (Corrodi *et al.*, 1973), in the same way as levodopa and dopamine agonists such as apomorphine and piribedil (Ungerstedt, 1971; Corrodi, Fuxe & Ungerstedt, 1971). There is substantial evidence that dopamine mediates hypothermia in rats placed in a cold ambient temperature (Kruk, 1972; Yehuda & Wurtman, 1972), so the findings reported here provide further support for the view that bromocriptine is a dopamine receptor agonist.

We wish to thank the Medical Research Council for supporting this work, and Sandoz for the supply of bromocriptine.

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SP1 Cardiovascular system
SP2 Central nervous system
SP3 Other physiological systems

Pharmacokinetics
PK1 Storage, distribution, fate
PK2 Synthesis, metabolism
PK3 Uptake, release

Autopharmacology
AP1 Adrenergic, cholinergic transmission
AP2 Histamine, kinins, prostaglandins, etc.
AP3 Hormones

Drug Mechanisms
DM1 Molecular, cellular, biochemical, biophysical
DM2 Receptors, antagonism, potentiation, tolerance

CP **Clinical Pharmacology, Experimental Therapy**
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FACTORS AFFECTING THE CEREBROVASCULAR RESPONSE TO NORADRENALINE IN THE DOG

I.M. JAMES & LINDSAY MACDONELL

Section of Clinical Pharmacology,* Departments of Medicine and Pharmacology,
The Royal Free Hospital, London WC1X 8LF

1 Noradrenaline infused into the internal carotid artery of the dog (0.01-1 $\mu g\ kg^{-1}\ min^{-1}$) constricts the blood vessels of the cortex. This constriction is mediated by the action of noradrenaline on α-adrenoceptors of the cerebral arteries.

2 Intravenous (1 $\mu g\ kg^{-1}\ min^{-1}$) or intra common carotid arterial (0.01-1 $\mu g\ kg^{-1}\ min^{-1}$) infusions of noradrenaline cause an increase in cortical blood flow that can be dissociated from changes in blood pressure.

3 The effect of intravenous noradrenaline on the cortical blood vessels and metabolism is blocked by high $Pa\mathrm{CO}_2$ levels, or by the prior administration of (±)-propranolol. (+)-Propranolol is without such effect.

4 Following section of both vagi and both sinus nerves, intravenous noradrenaline fails to cause an increase in cortical blood flow.

5 In another series of animals the area of the carotid bifurcation was vascularly isolated and perfused with blood from a second dog. Chemoreceptor and baroreceptor activity was shown to be intact.

6 Administration of 5% CO_2 to the donor dog caused an increase in cerebral blood flow in the recipient dog.

7 Administration of intravenous noradrenaline (1.0 $\mu g\ kg^{-1}\ min^{-1}$) to the donor animal caused an increase in cerebral blood flow, cerebral O_2 and glucose utilization of the recipient.

8 Administration of 5% CO_2 and intravenous (−)-noradrenaline (1.0 $\mu g\ kg^{-1}\ min^{-1}$) caused a further increase in flow and metabolism.

9 This evidence suggests that the cerebrovasodilatation observed following intravenous noradrenaline is reflex and is triggered by chemoreceptor activity.

10 The evidence also suggests that the antagonism of the cortical dilatory effects of intravenous noradrenaline by raised $Pa\mathrm{CO}_2$ in the intact animal must be at a site different from the peripheral chemoreceptors.

Introduction

The cerebral vasculature, in contrast with that in other beds was until fairly recently considered to be almost entirely regulated by local chemical changes, e.g. $P\mathrm{CO}_2$ or pH (Kety, 1960). However, it has now been suggested that cerebral blood vessels are reflexly controlled and that the carotid body chemoreceptors and carotid sinus baroreceptors initiate many of the cerebrovascular responses to hypoxia, hypercapnia and hypotension (Ponte & Purves, 1974). It appears reasonably certain that cerebral vessels are innervated in a similar fashion to those in other vascular beds. There is a great deal of morphological (Dahl & Nelson, 1964; Lavrentieva, Mchedlishvili & Plechkova, 1968; Iwayama, 1970; Iwayama, Furness & Burnstock, 1970) and histochemical (Falck, Nielsen & Owman, 1968; Ohgushi, 1968; Kajikawa, 1969) evidence to show that in most species there is an adrenergic pathway originating in the superior cervical ganglion which is a constrictor. There is also a dilator pathway, which may be cholinergic, that is carried by the VIIth cranial nerve.

Stimulation of the cervical sympathetic nerve causes unequivocal vasoconstriction (James, Millar & Purves, 1969; Harper, Deshmukh, Rowan & Jennett, 1972; D'Alecy & Feigl, 1972).

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Noradrenaline has been demonstrated in the nerve terminals (Falck *et al.*, 1968; Ohgushi, 1968) and is therefore likely to be the transmitter involved in the vasoconstrictor response. Although noradrenaline causes constriction of cerebral arterioles *in vitro* (Nielsen & Owman, 1971) its action *in vivo* is most uncertain. An increase, a decrease and no change in cerebral blood flow have all been reported in a recent review (Carpi, 1972).

Ponte & Purves (1974) have demonstrated in a series of experiments on the baboon that stimulation of vascularly isolated carotid body chemoreceptors with venous blood invariably caused a rise in regional cerebral blood flow. This response was abolished if the VIIth cranial nerves, purported to carry vasodilator nerve fibres, were cut intracranially. Cerebral blood flow varied inversely with carotid sinus pressure when cerebral perfusion pressure was held constant.

In the dog noradrenaline has been shown to cause an increase in the afferent discharge from the carotid body chemoreceptors (Black, Comroe & Jacobs, 1972). Some of the changes observed in the cerebral circulation following noradrenaline administration could, therefore, not only be due to a direct effect on the vessel wall, or secondary to blood pressure changes but could possibly be caused by activation of the carotid body chemoreceptors.

Some years ago we demonstrated that hypercapnia attenuated the vasodilator response of the cerebral circulation to an isoprenaline infusion (Xanalatos & James, 1972). It seemed appropriate therefore to determine what factors could affect the cerebrovascular response to noradrenaline in addition to the obvious ones of dose and route.

Methods

Mongrel dogs of mean weight 15 kg (s.d. ± 2) were anaesthetized with sodium pentobarbitone in a dosage of 25 mg/kg body weight. Tracheostomy was carried out and the dogs were ventilated at constant rate and depth throughout the experiment with a Starling respirator.

The right femoral vein was catheterized with a polyethylene catheter, through which maintenance doses of sodium pentobarbitone and experimental drugs were administered. A similar catheter placed in the aorta via the right femoral artery enabled blood pressure and arterial blood samples to be obtained. The arterial blood pressure was monitored with a Bell and Howell transducer in conjunction with a Devices preamplifier and subunit and the mean obtained electronically.

Cerebral blood flow

Cerebral blood flow was measured by the method of Ingvar & Lassen (1962) using the intracarotid injection of ^{85}krypton. Because 99% of the emissions from ^{85}krypton are β-particles and since the maximum penetration of these in brain tissue is only 2.5 mm (Glass, Harper & Glover, 1962) recording is almost totally from the cerebral cortex.

The left superior thyroid artery was identified and a fine polyethylene catheter introduced into the common carotid artery via this vessel. The catheter was directed towards the heart for a distance of 2 cm, so that the krypton injected through it would be washed upwards by the carotid arterial flow. It was felt that better mixing would be obtained in this way.

Craniotomy and removal of the dura was carried out as suggested by Ingvar & Lassen (1962).

Sufficient ^{85}krypton gas dissolved in about 1 ml of 0.9% w/v NaCl solution (saline) was injected slowly over approximately 1 min so that a constant plateau of radioactivity was monitored over the left parietal region for at least 45 seconds. The maximum count rate was always a factor of at least fifty times background. A ratemeter time constant of 5 s was used, and the counting scale employed was either 0.3 K or 1.0 K. Cerebral blood flow was then obtained from the analysis of the first 100 s of the ensuing decay curve as suggested by Lassen (1959). Radioactivity was measured with an end window Mullard Geiger Muller tube of effective window diameter 9 mm which was placed in such a way that large vessels were not within the counting field (Ingvar & Lassen, 1962). The Geiger Muller counter was coupled to a rate meter (Ekco Electronics Type N 522). The saturation and decay curves were recorded on a two channel potentiometric recorder (Smiths Industries Servoscribe 2, Type T/821).

A fine saggital sinus catheter was placed in such a way that flow was not impeded but samples of cortical venous blood could be obtained for blood gas analysis.

Cerebral O_2 and glucose metabolism were calculated as the product of flow and the arteriovenous difference. In this calculation it was assumed that the superior saggital sinus drains blood only from the cortex. There is good evidence in the dog to justify this assumption (Hegedus & Shackleford, 1965). Oxygen content was measured by the method of Linden, Ledsome & Norman (1965) and glucose by a glucose oxidase method (Trinder, 1969). pH, $P\text{aO}_2$ and $P\text{aCO}_2$ were measured with appropriate radiometer electrodes. The values were corrected for changes in ambient temperature according to the normogram of Severinghaus, Stupfel & Bradley (1965).

Experimental design

A period of one hour was allowed to elapse between completion of the surgery and starting the experiment. In each animal three sets of resting control measurements were made, 15 min apart, before any experimental procedure was started. The design of individual groups of experiments was as follows:

1. *Noradrenaline and CO_2 administration* (7 dogs) The effect of 5% CO_2 administration for 15 min was first assessed. Then whilst the animal was breathing room air (–)-noradrenaline was infused intravenously at a dose of 1 μg per kilogram body weight per minute. Measurements were carried out after 15 min of infusion. The drug infusion was continued and 5% CO_2 in air was simultaneously administered for a further period of 15 minutes. The CO_2 was discontinued and after another 15 min a further set of values was recorded for noradrenaline alone.

2. *Propranolol and noradrenaline* (5 dogs) In the second series of experiments (±)-propranolol at a total intravenous dose of 0.33 mg/kg body weight was infused over a 30 min period. This amount of propranolol had been found in separate experiments to abolish the increase in heart rate caused by intravenous infusion of isoprenaline at a rate of 0.4 μg kg^{-1} min^{-1} for at least a 30 min period (MacDonell, 1974). This was the dose of isoprenaline used by Xanalatos & James (1972) (see discussion section). Measurements were made after 15 and 30 min of propranolol infusion. The propranolol was discontinued and noradrenaline was administered intravenously in a dose of 1 μg kg^{-1} min^{-1}. All measurements were repeated after this drug had been infused for 15 and 30 minutes.

In two additional dogs, the noradrenaline was infused following pretreatment with 0.33 mg/kg (+)-propranolol administered over 30 minutes. In one further animal, propranolol was replaced by 3.3 mg/kg practolol given over 30 minutes.

3. *Phenoxybenzamine and noradrenaline* (5 dogs) In the third group of experiments, phenoxybenzamine in a total intravenous dose of 0.67 mg/kg was infused over a 60 min period. Previous experiments (MacDonell, 1974) showed that this dose causes a 38% attenuation of the maximum pressor response to an intravenous infusion of noradrenaline (1.0 μg kg^{-1} min^{-1}). In other studies phenoxybenzamine has been used to 'block' α-adrenoceptors in the dog in doses ranging from 1.0 mg/kg to 10.0 mg/kg (Carr, Cooper, Daggett, Lish, Nugent & Powers, 1967; Olivares, Smith & Aronow, 1967). However, under the present experimental conditions doses larger than the one employed were found to cause a precipitous fall in blood pressure.

When the effect of the total dose of phenoxybenzamine had been recorded, noradrenaline was administered intravenously, as before (1.0 μg kg^{-1} min^{-1}).

4. *Common carotid noradrenaline administration* (7 dogs) Four doses of noradrenaline were infused into seven dogs via a fine catheter placed in the common carotid artery. The doses employed were. 0.01, 0.10, 0.50 and 1.00 μg kg^{-1} min^{-1}. Measurements were made after 15 min of infusion, and 30 min were allowed to elapse between termination of the lower dose and the start of the next higher dose.

5. *Internal carotid noradrenaline administration* (5 dogs) The left internal carotid artery was catheterized via the superior thyroid artery. Noradrenaline was infused through this in doses of 0.01, 0.10 and 1.00 μg kg^{-1} min^{-1} following the same regime as in the fourth series of experiments.

In five separate animals this procedure was repeated after a total dose of 0.067 mg/kg phenoxybenzamine had been infused into the left internal carotid artery, over a period of 30 minutes. It was assumed that approximately one tenth of the cardiac output passes up one carotid artery and hence the dose of phenoxybenzamine was one tenth of that administered intravenously in the previous experiments.

6. *Intravenous noradrenaline administration following afferent denervation* (6 dogs) Both sinus nerves and both vagosympathetic trunks were sectioned in six dogs. Afferent denervation was assumed to be complete if there was no blood pressure response to occlusion of the common carotid artery. Noradrenaline was infused intravenously in a dose of 1.0 μg kg^{-1} min^{-1}.

7. *Vascular isolation of carotid bifurcation area: effect of noradrenaline* Experiments were carried out each of which involved two dogs. The carotid bifurcation regions of one dog (the recipient) were vascularly isolated and perfused with arterial blood from a second dog (the donor) (Figure 1). The carotid bifurcations were exposed bilaterally in the recipient dog. The internal carotid, the external carotid above the junction of the lingual artery

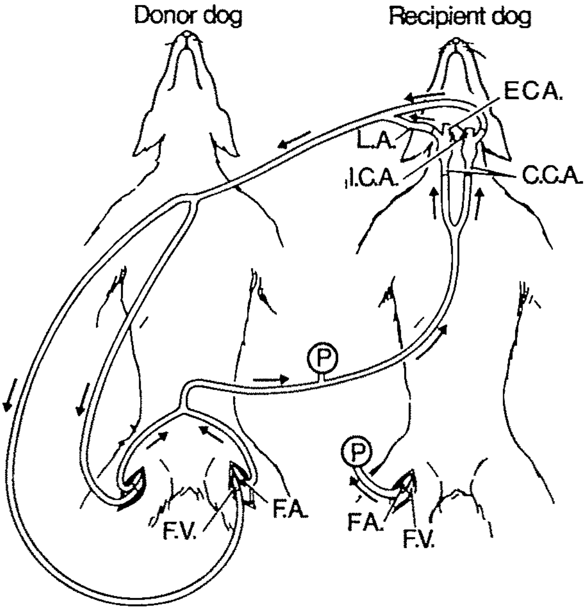

Figure 1 Final preparation involving two dogs. In the recipient dog both vagosympathetic nerve trunks had been divided. Both sinus nerves are intact. Internal carotid arteries bilaterally occluded. Brain is perfused by vertebral and many other anastomotic arteries. F.V. = femoral vein, F.A. = femoral artery, P = blood pressure transducer, I.C.A. = internal carotid artery, C.C.A. = common carotid artery, E.C.A. = external carotid artery, L.A. = lingual artery.

and all other branches such as the occipital artery of the first dog were ligated.

Blood was initially shunted from both femoral arteries into the common carotid arteries and then back into the femoral veins via catheters in the lingual arteries. Great care was taken to ensure that the sinus nerves were not damaged during this dissection. To eliminate aortic receptor responses, bilateral vagotomy was performed. The sympathetic supply to the head was also partially interrupted as this portion of the sympathetic runs with the vagus as a single trunk in the dog (Stromberg, 1964).

As soon as the remaining surgical procedure necessary for measuring cortical blood flow had been completed (see below) the donor dog was anaesthetized and tracheostomized as before. This dog was also ventilated at a constant rate and depth throughout the experiment.

Both femoral arteries and femoral veins were catheterized. Femoral arterial blood was then shunted from the donor dog into the common carotid arteries of the recipient dog. This blood was returned to the femoral veins of the donor dog via the lingual arteries of the recipient dog.

During the switching over of the blood supply to the recipient carotid bifurcation regions from recipient to donor dog, the time that this region was without a blood supply was limited to 30 seconds.

To prevent clotting of blood in the recipient carotid bifurcation areas, the donor dog was injected with 25,000 units of heparin.

The systematic arterial blood pressure of the recipient dog, and the perfusion pressure of the vascularly isolated carotid bifurcation regions were monitored with Bell and Howell pressure transducers and electronically meaned.

Cerebral blood flow was measured as before but some minor modification of the method was necessary. On this occasion the ^{85}krypton reached the parietal region via the right vertebral artery. A catheter was inserted in a retrograde manner from the superior thyroid artery on the right into the brachiocephalic artery and the gas dissolved in saline was injected as before over approximately a 1 min period so that a constant amount of radioactivity monitored over the parietal region was obtained for at least 45 seconds.

Once the preparation was complete, the following procedures were carried out: (a) At least two control measurements were recorded. (b) Five per cent CO_2 in air was administered to the donor dogs to inhale for 15 min (10 dogs).

(c) Noradrenaline in a dose of 1.0 μg kg^{-1} min^{-1} was infused intravenously into the donor dog (6 dogs). Perfusion pressure was kept constant and measurements were made at the end of a 15 min period. (d) During the continuing noradrenaline infusion, 5% CO_2 in air was simultaneously administered to the donor dog for a further 15 min period (6 dogs). Perfusion pressure was kept constant.

Drugs and chemicals

These included (−)-noradrenaline bitartrate (Koch Light Laboratories), (±)-propranolol hydrochloride and (+)-propranolol hydrochloride (ICI), phenoxybenzamine hydrochloride.

The doses used were expressed in terms of the respective salts. All drugs were dissolved in saline and were administered as above.

Results

1. *Noradrenaline and CO_2 administration*

Control values (Table 1) The mean resting value of cortical blood flow in the seven animals was 108.6 ± 9.0 (s.e. mean) ml per 100 g of cortical tissue per minute.

Mean cortical O_2 consumption was 10.9 ± 1.1 (s.e. mean) ml per 100 g per min and mean cortical glucose consumption was 16.9 ± 2.4 (s.e. mean) mg per 100 g per minute.

Mean control values of P_{aCO_2}, arterial hydrogen ion concentration and mean arterial blood pressure were 38.7 ± 2.6 mmHg, 50.9 ± 2.5 nmol per litre and 144.0 ± 8.6 mmHg respectively.

These values were similar to those previously obtained from this laboratory (Xanalatos & James, 1972).

i. *Inhalation of 5% CO_2 in air* The inhalation of 5% CO_2 for a period of 15 min caused an increase in cortical blood flow in all seven dogs (Table 1). The increase in arterial CO_2 tension had no statistically significant effect upon cortical oxygen consumption, although the mean value was slightly depressed from control. Cortical glucose consumption was observed to be lower than control levels after 15 min of CO_2. The mean reduction was 6.9 mg per 100 g per minute. After the CO_2 administration had been discontinued, all the variables measured returned to previous values by the end of 15 minutes.

ii. *Intravenous infusions of noradrenaline* (Table 1) Noradrenaline was administered by con-

Table 1 Effect of 5% CO_2, noradrenaline (1 μg kg^{-1} min^{-1}) and noradrenaline plus CO_2 in the dog

	Control	*5% CO_2*	*Control*	*Noradrenaline*	*Noradrenaline +5% CO_2*	*Noradrenaline*
Cortical blood flow (ml 100 g^{-1} min^{-1})	108.6 ±9.0	147.9* ±10.2	107.1 ±9.6	138.7* ±13.5	92.6*+ ±8.0	135.3* ±8.6
Cortical O_2 consumption (ml 100 g^{-1} min^{-1})	10.9 ±1.1	9.9* ±0.8	11.3 ±1.3	15.2* ±2.0	7.2*+ ±0.6	14.7* ±0.8
Cortical glucose consumption (mg 100 g^{-1} min^{-1})	16.9 ±2.4	9.9* ±0.7	16.3 ±2.1	30.7* ±3.6	15.1+ ±4.0	31.0* ±6.0
Arterial CO_2 tension (mmHg)	38.7 ±2.6	59.9* ±3.0	38.4 ±2.5	41.9* ±2.9	62.7*+ ±3.1	43.7 ±3.1
Arterial hydrogen ion concentration (nmol/litre)	50.9 ±2.5	65.5* ±2.3	52.2 ±2.5	55.4* ±2.7	74.2*+ ±2.3	58.5* ±3.0
Mean arterial blood pressure (mmHg)	144.0 ±8.6	149.7* ±7.1	145.0 ±7.5	149.3 ±7.4	145.4 ±6.8	146.4 ±4.7

All measurements made at the end of a 15 min period. All P_{aO_2} measurements were above 100 mmHg. Means ± s.e. mean are shown. $n = 7$.

* Significantly different from 1st control by paired analysis at 5% level.

+ Significantly different from preceding noradrenaline levels by paired analysis at 5% level.

tinuous intravenous infusion in a dose of 1.0 μg/kg body weight per minute. In all experiments, at the end of 15 min of noradrenaline infusion cortical blood flow was higher than resting control values. Both cortical O_2 and cortical glucose consumption were increased, although the elevation in O_2 consumption was not as great as that in glucose consumption. Blood pressure, which was initially elevated by the noradrenaline infusion, had returned to within control levels after 15 min of infusion and was thus at this time not significantly different from control values (Table 1).

iii. *Noradrenaline and simultaneous 5% CO_2* (Table 1) Five per cent CO_2 in air was then readministered during the continuing noradrenaline infusion for a period of 15 minutes. Combined noradrenaline and CO_2 caused a fall in cortical blood flow to a mean value of 93 ml per 100 g per minute, which was lower than not only the initial noradrenaline level but also the initial control values.

Cortical O_2 and glucose consumptions both fell from their previously elevated levels. Oxygen utilisation was also lower than the control levels, but glucose uptake fell only to resting values.

The fall in blood flow occurring at a time when arterial blood pressure was constant suggests that cortical vasoconstriction was taking place.

The administration of CO_2 was then terminated, but the infusion of noradrenaline continued. After 15 min, all the variables measured returned to those values observed when noradrenaline had previously been administered alone. This suggested that the changes occurring with noradrenaline plus CO_2 were not due to the increasing cumulative dose of noradrenaline, but due to the addition of CO_2.

2. *Propranolol and noradrenaline* (Table 2)

a. *Effect of propranolol* At the end of the propranolol infusion, cortical blood flow, cortical O_2 and glucose consumption and mean arterial blood pressure were all significantly lower than control values.

b. *Effect of noradrenaline after propranolol* (Table 2) As soon as the final propranolol measurements had been made, noradrenaline in the same dose as in the previous experiments (1.0 μg kg^{-1} min^{-1}) was infused intravenously. After 15 min of infusion, a further fall in both cortical blood flow and O_2 uptake was seen (Figure 2). Cortical glucose consumption was more variable, but tended to remain low.

The fall in mean arterial blood pressure due to propranolol was reversed by noradrenaline. The

Table 2 Effect of propranolol (total dose 0.33 mg/kg given i.v. over 30 min) and noradrenaline (1.0 μg kg^{-1} min^{-1}, i.v.) immediately following propranolol in dog.

	Control	*Control*	*Propranolol*	*Propranolol*	*Noradrenaline*	*Noradrenaline*
Cortical blood flow ($ml\ 100\ g^{-1}\ min^{-1}$)	98.6 ±7.2	99.8 ±7.3	74.4* ±10.8	76.6* ±6.8	56.8* ±4.3	77.0* ±7.3
Cortical O_2 consumption ($ml\ 100\ g^{-1}\ min^{-1}$)	10.6 ±0.9	10.7 ±1.1	8.3* ±1.2	9.3* ±0.9	7.0* ±0.7	10.7 ±1.1
Cortical glucose consumption ($mg\ 100\ g^{-1}\ min^{-1}$)	15.4 ±1.3	15.7 ±1.4	12.9 ±2.3	11.0* ±0.7	11.6* ±1.6	14.1* ±1.3
Mean arterial blood pressure (mmHg)	127.0 ±13.2	125.6 ±14.9	117.6* ±13.4	108.2* ±11.4	126.4 ±12.2	123.4 ±12.2
Arterial hydrogen ion concentration (nmol/litre)	46.8 ±2.6	46.1 ±2.3	44.4 ±2.8	44.2 ±2.2	46.2 ±3.1	48.4 ±4.1
$PaCO_2$ (mmHg)	32.6 ±2.9	32.2 ±2.9	30.4 ±3.7	30.4 ±3.6	30.8 ±3.4	31.2 ±3.3

All measurements made at the end of 15 min periods. All PaO_2 measurements above 100 mmHg. Means ± s.e. mean are shown. $n = 5$.

* Significantly different from 1st control values by paired analysis at the 5% level.

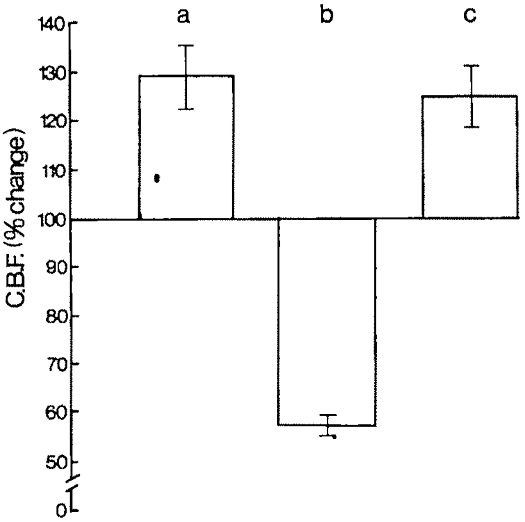

Figure 2 Changes in cortical blood flow in response to 15 minutes of intravenous infusion of noradrenaline (1.0 μg kg^{-1} min^{-1}) in dog. (a) After no pretreatment (n = 7), (b) after pretreatment with propranolol (0.33 mg/kg) (n = 5), (c) after pretreatment with phenoxybenzamine (0.67 mg/kg) (n = 5). Cerebral cortical blood flow (C.B.F.) expressed as percentage of control (= 100%). Vertical bars show s.e. mean.

fall in blood flow occurring at a time when arterial blood pressure increased suggests that cortical vasoconstriction was taking place.

After 30 min of noradrenaline infusion cortical flow, O_2 and glucose consumption were seen to increase back towards control levels. Both flow and glucose utilization were still somewhat depressed, but O_2 consumption was no longer significantly different from control.

c. *Effect of noradrenaline after (+)-propranolol* In an attempt to establish whether the observed effect of propranolol on the response by cortical blood flow and metabolism to noradrenaline was due to its β-adrenoceptor blocking properties or to its membrane stabilizing activity, two further experiments were carried out. In these, noradrenaline was administered intravenously in the same dose as before following pretreatment with (+)-propranolol. This drug, which has little β-adrenoceptor blockading activity, but has similar local anaesthetic properties to those of (±)-propranolol (Howe & Shanks, 1966) was given in an intravenous dose of 0.33 mg/kg over a 30 min period. Pretreatment with this drug failed to cause any changes in flow or metabolism or markedly alter the response to the intravenous administration of noradrenaline.

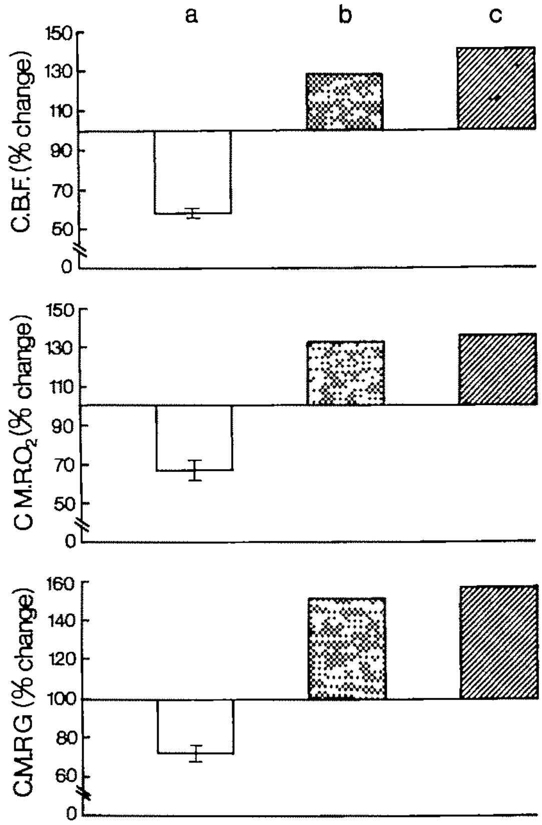

Figure 3 Effect of noradrenaline (1.0 μg kg^{-1} min^{-1}, 15 min intravenous infusion) on cortical blood flow, cortical O_2 consumption and cortical glucose consumption expressed as a percentage change from control (= 100%). (a) After pretreatment with (±)-propranolol (0.33 mg/kg) (n = 5), (b) after pretreatment with (+)-propranolol (0.33 mg/kg, average of 2 experiments), (c) after pretreatment with practolol (3.3 mg/kg), 1 experiment. C.B.F. = cortical blood flow, C.M.R.O_2 = cortical O_2 consumption, C.M.R.G. = cortical glucose consumption. Vertical bars show s.e. mean.

The changes observed after 15 min of intravenous noradrenaline following prior administration of (+)-propranolol are represented in Figure 3.

These results, although from only two experiments, suggest that membrane stabilization was not responsible for the altered cerebrovascular response to intravenous noradrenaline following treatment with (±)-propranolol.

d. *Effect of noradrenaline after practolol* Another experiment was carried out, this time practolol was given before noradrenaline. Practolol, besides being a selective blocker of

β_1-adrenoceptors (Dunlop & Shanks, 1968) does not enter the brain to any great extent (Estler & Ammon, 1969). Because propranolol readily passes the blood brain barrier (Laverty & Taylor, 1968) it was thought possible that the observed action of this drug could be centrally mediated.

In clinical practice, propranolol has been used in a dose approximately one tenth of that used with practolol (Meier, 1972). Hence, in the present study, 3.3 mg/kg practolol (ten times the dose of (±)-propranolol used) was administered intravenously over a 30 min period. Pretreatment with practolol, like (+)-propranolol, failed to alter markedly the cerebrovascular response to an intravenous infusion of noradrenaline (Figure 3).

3. *Phenoxybenzamine and noradrenaline* (Table 3)

a. *Effect of phenoxybenzamine (0.67 mg/kg)* This intravenous dose of phenoxybenzamine caused a substantial drop in mean arterial blood pressure. For this reason the drug was given slowly over a period of 1 h in an attempt to block cerebrovascular α-adrenoceptors without causing a precipitous fall in blood pressure.

The fall in blood pressure was usually accompanied by a small decrease in cortical blood flow. There was no consistent change in mean cortical O_2 or glucose consumption. In one of the five animals, phenoxybenzamine caused a decrease in cortical O_2 consumption whereas in the remaining four an increase occurred. Similarly, in another dog, phenoxybenzamine caused a substantial fall in cortical glucose consumption, whereas in the remaining four this variable increased.

b. *Noradrenaline following phenoxybenzamine* (Table 3) After intravenous infusion of noradrenaline in the usual dose of 1.0 μg per kg per min for 15 min, both cortical blood flow and cortical O_2 consumption were significantly elevated above control values (Figure 2).

Changes in cortical glucose consumption were, however, more variable. In three animals this was higher than controls at this time, but in two, glucose utilization fell.

Mean arterial blood pressures increased back towards control values, but this change was less than that in flow, suggesting that noradrenaline after phenoxybenzamine caused cortical vasodilation.

The values for cortical blood flow and metabolism increased again slightly during the continuing infusion of noradrenaline for another 15 min period, and these values were then all statistically different from control levels.

Mean arterial blood pressure failed to show any further consistent change.

4. *Common carotid noradrenaline administration*

Cortical O_2 and glucose consumption were not measured because the noradrenaline was infused unilaterally. Venous samples for metabolism recordings have to be taken from the superior saggital sinus, which drains both sides of the cerebral cortex, meaning that metabolism measurements following unilateral administration of a drug would not be representative of metabolic changes in the area of cortex over which flow was measured.

Thus, in this group of experiments, as in the next series, flow was measured on the same side as

Table 3 Effect of phenoxybenzamine (total dose 0.67 mg/kg infused i.v. over one hour) and noradrenaline (1.0 μg kg^{-1} min^{-1}, i.v.) following phenoxybenzamine in the dog

	Control	*Control*	*Phenoxybenzamine*	*Noradrenaline*	*Noradrenaline*
Cortical blood flow (ml 100 g^{-1} min^{-1})	88.2 ±7.5	88.4 ±8.2	77.4* ±8.1	109.8* ±9.9	114.8* ±8.8
Cortical O_2 consumption (ml 100 g^{-1} min^{-1})	8.8 ±0.8	8.8 ±0.9	9.6 ±1.2	13.1* ±1.8	15.2* ±1.9
Cortical glucose consumption (mg 100 g^{-1} min^{-1})	13.2 ±2.0	13.0 ±1.4	13.6 ±2.0	19.6 ±3.5	23.2* ±2.2
Mean arterial blood pressure (mmHg)	132.8 ±8.5	132.8 ±8.1	105.0* ±5.7	111.0* ±5.2	112.2* ±5.9
Arterial $P\text{CO}_2$ (mmHg)	37.4 ±1.9	36.6 ±2.4	36.0 ±2.4	38.6 ±3.3	38.8 ±3.1

All measurements (except those at the end of the phenoxybenzamine infusion) were made at the end of 15 min periods. All $P\text{aO}_2$ measurements above 100 mmHg. Means ± s.e. mean are shown. $n = 5$.

* Significantly different from 1st control values by paired analysis at the 5% level.

the infusions of noradrenaline. The results are shown in Table 4.

a. *Infusions of 0.01 μg kg^{-1} min^{-1}* Noradrenaline infused into the common carotid artery at this dose for 15 min, caused no significant change in cortical blood flow or systemic arterial blood pressure. In three of the seven experiments, however, cortical blood flow fell, but in only two by large amounts.

b. *Infusions of 0.1 μg kg^{-1} min^{-1}* No consistent change followed these doses of noradrenaline. On two occasions cortical blood flow increased, but in the remaining five experiments there was a tendency for flow to fall. No significant change in mean arterial blood pressure occurred.

c. *Infusions of 0.5 μg kg^{-1} min^{-1}* At the end of the 15 min infusion of noradrenaline at this dose, there was a significant increase in cortical blood flow. Mean arterial blood pressure did not significantly change.

d. *Infusions of 1.0 μg kg^{-1} min^{-1}* At this highest dose used, cortical blood flow was again significantly raised above control values. The mean value was, however, somewhat lower than with the previous dose of 0.5 μg kg^{-1} min^{-1}.

All values returned to within resting levels during the 30 min control periods between each dose.

5. *Internal carotid noradrenaline administration* (Table 5)

i. *Infusion of 0.01 μg kg^{-1} min^{-1}* This dose of noradrenaline into the internal carotid artery caused a small decrease in cortical blood flow and no significant change in mean arterial blood pressure (Figure 4).

ii. *Infusion of 0.1 μg kg^{-1} min^{-1}* A decrease in cortical blood flow occurred at the end of 15 min of this dose of noradrenaline infused into the internal carotid artery. This fall in flow was greater than observed with the previous dose. Mean arterial blood pressure was not significantly changed from control levels (Figure 4).

iii. *Infusions of 1.0 μg kg^{-1} min^{-1}* These relatively large doses of noradrenaline directly into the internal carotid artery caused a similar reduction in cortical blood flow to that seen with

Table 4 Effect of infusions of noradrenaline into the left common carotid artery of the dog.

	Common carotid noradrenaline infusions (μg kg^{-1} min^{-1})				
	Control	*0.01*	*0.10*	*0.50*	*1.00*
Cortical blood flow	88.1	82.3	79.0	120.4*	108.7*
(ml 100 g^{-1} min^{-1})	±12.1	±12.8	±14.6	±16.5	±16.0
Mean arterial blood pressure	133.4	134.4	133.4	134.4	133.3
(mmHg)	±6.5	±6.8	±6.1	±5.8	±6.6

Each dose was infused for 15 min at the end of which the measurements were made. A 30 min control period (not shown) was allowed to elapse between the different doses. All PaO_2 measurements above 100 mmHg. Means ± s.e. mean are shown. $n = 7$ (for each dose).

* Significantly different from 1st control by paired analysis at 5% level.

Table 5 Effect of noradrenaline infused for 15 min into the left internal carotid artery of the dog

	Internal carotid noradrenaline infusions (μg kg^{-1} min^{-1})				
	Control	*0.01*	*0.10*	*1.00*	*Control*
Cortical blood flow	74.2	64.4*	50.0*	50.4*	76.0
(ml 100 g^{-1} min^{-1})	±8.6	±7.1	±6.3	±7.2	±8.0
Mean arterial blood pressure	138.4	130.5	136.6	151.0	137.6
(mmHg)	±5.1	±4.3	±5.4	±9.4	±3.6

All PaO_2 measurements above 100 mmHg. Means ± s.e. mean are shown. $n = 5$.

* Significantly different from 1st control by paired analysis at 5% level.

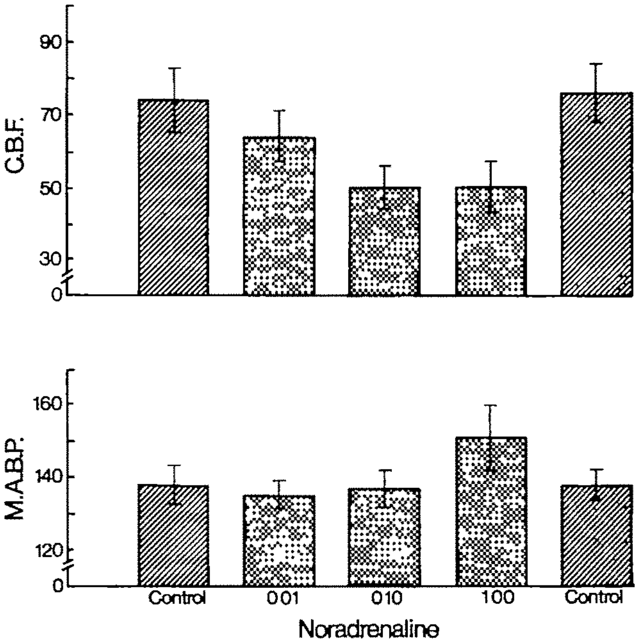

Figure 4 Effect on cortical blood flow and mean arterial blood pressure of increasing doses of noradrenaline infused for 15 min into the left internal carotid artery of the dog. Doses given in μg kg^{-1} min^{-1}. C.B.F. = cortical blood flow (ml 100 g^{-1} min^{-1}). M.A.B.P. = mean arterial blood pressure (mmHg). Vertical bars show s.e. mean, $n = 5$.

the previous dose of 0.1 μg kg^{-1} min^{-1}. There was a tendency for mean arterial blood pressure to increase.

All values returned to within control levels during the 30 min control period following termination of each infusion of each dose used.

Internal carotid noradrenaline infusions after pretreatment with phenoxybenzamine The internal carotid arterial infusions of noradrenaline were repeated in another five dogs after phenoxybenzamine 0.067 mg/kg had been infused during 30 min (Table 6).

After control measurements, cortical blood flow was recorded at the end of the phenoxybenzamine infusion, and after 15 min of noradrenaline infusions (0.01, 0.10 and 1.00 μg kg^{-1} min^{-1}). As before, a 30 min control period was allowed between the different noradrenaline doses. During this time, all the variables measured returned to within their internal resting values.

As can be seen from Figure 5, phenoxy-

Table 6 Effect of phenoxybenzamine (0.067 mg/kg) infused into the left internal carotid artery, and of 15 min infusions of noradrenaline into the left internal carotid artery after phenoxybenzamine in the dog

				Internal carotid noradrenaline (μg kg^{-1} min^{-1})		
	Control	*Control*	*Phenoxybenzamine*	*0.01*	*0.10*	*1.00*
Cortical blood flow (ml 100 g^{-1} min^{-1})	85.2 ±7.6	85.8 ±8.2	90.2* ±7.7	90.6 ±7.6	87.0 ±7.3	89.0 ±7.2
Mean arterial blood pressure (mmHg)	131.8 ±10.1	132.6 ±9.9	129.8 ±11.5	131.4 ±11.9	130.8 ±12.7	132.8 ±12.9

All PaO_2 measurements above 100 mmHg. Means ± s.e. mean are shown. $n = 5$.

* Significantly different from 1st control by paired analysis at 5% level.

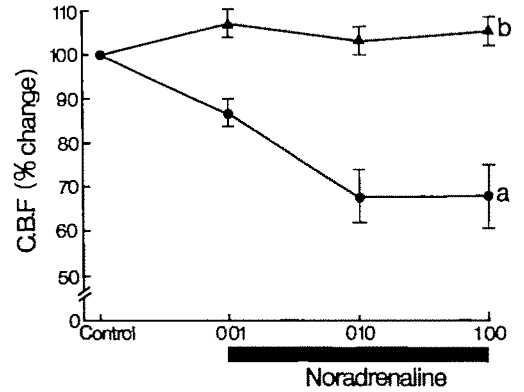

Figure 5 Changes in cortical blood flow during 15 min infusions of increasing doses of noradrenaline (0.01, 0.10 and 1.00 μg kg^{-1} min^{-1} into the left internal carotid artery. (a) Following no pretreatment (●) $n = 5$, (b) following pretreatment with phenoxybenzamine (1.0 mg infused into the internal carotid artery over a period of 30 min) (▲) $n = 5$. C.B.F. = cortical blood flow expressed as percentage changes from control (= 100%). Vertical bars show s.e. mean.

benzamine completely abolished the previously observed cortical vasoconstrictor action of all the doses of noradrenaline given directly into the internal carotid artery. These results suggest that if noradrenaline is administered directly into the internal carotid artery, an α-adrenoceptor mediated vasoconstriction occurs in the cerebral cortex.

6. *Intravenous noradrenaline following afferent denervation*

Noradrenaline infused intravenously at a dose of 1.0 μg kg^{-1} min^{-1} for 30 min caused no significant change in cortical blood flow. It should be noted however, that the initial flow values were low.

It was noticed that in these preparations, the blood pressure response to noradrenaline became attenuated more slowly than previously observed. Thus in some experiments blood pressure was still elevated well above control values even after 30 min of noradrenaline infusion.

7. *Effect of noradrenaline in cross perfusion experiments*

Baroreceptor and chemoreceptor reflexes To ensure that the sinus nerves had not been damaged during the preparation, the following tests were carried out:

The perfusion pressure in the system supplying the vascularly isolated carotid bifurcation area of the recipient dogs was raised and then lowered. This was done by partially occluding the outflow and inflow catheters to the system respectively, by means of a screw clip. The change, in the opposite direction, of the recipient systemic arterial blood pressure was taken to indicate that the carotid baroreceptors were functioning normally (Heymans & Neil, 1958).

The recipient dogs were allowed to ventilate spontaneously for a time and resting respiratory rate was monitored; 5% CO_2 was then administered to the donor dog. The resulting increase in respiratory rate and decrease in arterial $P\text{CO}_2$ of the recipient animal was assumed to demonstrate that this dog's carotid body chemoreceptors were also functioning normally (Biscoe, Purves & Sampson, 1970).

a. *Control values* At least two control values were recorded before each experimental procedure. The mean control cortical blood flow was 66.1 (s.e. mean ± 5.0) ml 100 g^{-1} min^{-1}. Cortical O_2 consumption was (s.e. mean ± 0.8) ml 100 g^{-1} min^{-1}, and cortical glucose consumption was 11.3 (s.e. mean ± 1.3) mg 100 g^{-1} min^{-1}.

During most procedures there was no significant change in A-V O_2 or glucose differences, hence changes in cortical metabolism were qualitatively similar to those in flow.

Table 7 The effect of noradrenaline (1.0 μg kg^{-1} min^{-1}) for 15 and 30 min following bilateral vagus and sinus nerve section in the dog

	Control	*Control*	*Noradrenaline*	*Noradrenaline*	*Control*
Cortical blood flow (ml 100 g^{-1} min^{-1})	55.8 ±11.8	59.0 ±13.8	69.7 ±12.3	67.5 ±13.3	52.8 ±11.0
Mean arterial blood pressure (mmHg)	127 ±6.5	129 ±5.8	165* ±6.1	147 ±10.1	117 ±16.5

All $P\text{aO}_2$ measurements above 100 mmHg. $n = 6$.
* Significantly different from 1st control by paired analysis at 5% level.

Table 8 Cross perfusion experiments. Effect on recipient dog of: (a) two consecutive control periods, (b) administration of 5% CO_2 to donor dog, (c) noradrenaline (1 μg kg^{-1} min^{-1}, i.v.) administered to donor dog for 15 min, (d) noradrenaline (1 μg kg^{-1} min^{-1}, i.v.) plus 5% CO_2 administered to donor dog for 15 minutes

	C.B.F.		*M.A.B.P.*		*P.P.*		$PaCO_2$		*C.M.R.O₂*		*C.M.R.G.*	
	C	*C*	*C*	*C*	*C*	*C*	*C*	*C*	*C*	*C*	*C*	*C*
a. Control	66.1 ±5.0	64.3 ±5.6	124 ±10.2	122 ±11.2	96 ±9.6	94 ±10.0	38.1 ±2.7	38.7 ±3.2	9.0 ±0.8	8.7 ±0.7	11.3 ±1.3	11.0 ±1.2
	C	*Exp*	*C*	*Exp*	*C*	*Exp*	*C*	*Exp*	*C*	*Exp*	*C*	*Exp*
b. 5% CO_2 to donor dog	67.9 ±5.0	91.7* ±7.9	123.5 ±10.2	122.0 ±10.1	95.8 ±9.6	94.0 ±7.8	42.4 ±3.3	40.3 ±3.2	9.0 ±0.8	13.3* ±1.3	11.3 ±1.3	17.1* ±2.2
c. Noradrenaline	55.3 ±2.7	83.0* ±5.1	105.7 ±17.0	111.2 ±19.5	101.8 ±9.3	108.5 ±11.8	37.2 ±4.2	38.0 ±4.4	7.9 ±0.7	11.6* ±1.8	9.5 ±1.6	16.3* ±1.7
d. Noradrenaline + 5% CO_2	75.8 ±5.8	89.3* ±5.0	104.3 ±13.3	109.8 ±15.4	106.7 ±12.5	110.7 ±10.6	38.0 ±3.7	37.7 ±4.3	10.8 ±1.5	12.9* ±1.7	14.7 ±1.9	17.2 ±2.3

For procedures b, c and d, experimental values are compared with immediately preceding control values. Means ± s.e. mean are shown. n = 10 for (a) and (b); n = 6 for (c) and (d).

* Significantly different from preceding control by paired analysis ($P < 0.05$).

C = control, Exp = experimental, C.B.F. = Cortical blood flow (ml 100 g^{-1} min^{-1}), C.M.R.O_2 = Cortical O_2 consumption (ml 100 g^{-1} min^{-1}), M.A.B.P. = mean arterial blood pressure of recipient dog (mmHg), P.P. = perfusion pressure to recipient carotid bifurcation areas (mmHg), $PaCO_2$ = arterial CO_2 tension in recipient dog (mmHg). All measurements of PaO_2 in recipient and donor dogs above 100 mmHg.

b. *CO_2 (5%) to donor dog* After 15 min of CO_2 administration to the donor dog, recipient cortical blood flow, O_2 and glucose consumption were significantly elevated above control values.

There was no significant change in recipient systemic arterial blood pressure.

c. *Noradrenaline to donor dog* The intravenous infusion of noradrenaline to the donor dog consistently caused an increase in cortical blood flow and metabolism in all of the six experiments. There was no significant change in recipient systemic arterial blood pressure after 15 minutes. Perfusion pressure was kept constant despite an initial rise in the blood pressure of the donor dog.

d. *Noradrenaline plus CO_2 to donor dog* A further increase in cortical blood flow and O_2 consumption above the already elevated noradrenaline levels was seen in these experiments. A further, small increase in cortical glucose consumption was also observed.

There was no change in recipient $PaCO_2$. Systemic arterial blood pressure of the recipient dog was not markedly altered, since carotid perfusion pressure was kept constant.

Discussion

The average error in the measurement of cerebral blood flow by this method has previously been shown by us to be in the region of 5% (s.d. ± 3.1%) (MacDonell, 1974). The variation in the control flows between various groups of animals studied cannot be ascribed therefore to this. Differences in age of the animals and source of supply are probably more important factors.

Inhalation of 5% CO_2 gave rise to an increase in cerebral blood flow, a small decrease in O_2 consumption and a larger decrease in cerebral glucose consumption.

The changes in cerebral blood flow, O_2 and glucose consumption during noradrenaline infusion at normal and raised CO_2 levels are very similar to those reported by us as occurring with the β-agonist, isoprenaline (Xanalatos & James, 1972). The increase in flow is unlikely to be due to an increase in blood pressure. The blood pressure rise was very largely attenuated following fifteen minutes of noradrenaline infusion.

Both isoprenaline 0.4 μg kg^{-1} min^{-1} and noradrenaline 1.0 μg kg^{-1} min^{-1} caused similar increases in O_2 consumption. However, there was not such a large rise in flow with noradrenaline as isoprenaline. When CO_2 was administered during the isoprenaline infusion, cerebral blood flow fell to control levels, whereas when given during the noradrenaline infusion, cerebral blood flow fell to values significantly below control.

Since it has been shown that many β-adrenergic effects are abolished or attenuated by administration of CO_2 (Schroeder, Robinson, Miller & Harrison, 1970; Xanalatos & James, 1972) and also that some of the effects of noradrenaline (such as those on the heart) are β-effects (Benfey & Varma, 1964; Brick, Hutchinson & Roddie, 1966a,b) it was decided that the effect of noradrenaline infusion following the prior administration of the β-adrenoceptor blocking agent, propranolol, should be evaluated.

The blood pressure fell by 15% during the propranolol infusion, but had returned to control levels by the time the noradrenaline was administered.

The propranolol caused a slight fall in cerebral blood flow and O_2 consumption and a greater fall in glucose consumption. When noradrenaline was given intravenously a clear fall in cerebral blood flow was observed. This was accompanied by a further small fall in cerebral O_2 consumption, but glucose consumption remained at the same level.

Under these circumstances it appeared, therefore, that once the vasodilator effects of noradrenaline were attenuated, cerebral vasoconstriction ocurred.

(+)-Propranolol did not have any significant effect on its own or on the noradrenaline response. It is therefore likely that response to (±)-propranolol was due to its adrenoceptor blocking properties rather than its membrane stabilizing ones.

The administration of practolol in appropriate doses also failed to alter the response. Whether this was due to the fact that practolol is a water soluble drug and penetrates the blood brain barrier poorly or principally has only β_1-adrenoceptor blocking properties is unclear.

The effect of phenoxybenzamine on brain metabolism was variable but usually caused a small decrease in cortical blood flow and a substantial drop in mean arterial pressure. Noradrenaline administration following phenoxybenzamine resulted in the usual increase in flow and metabolism.

The observed increases in cortical blood flow during infusions of noradrenaline into the common carotid artery were similar to those obtained with intravenous noradrenaline. This suggests that the mechanisms involved in the cortical vasodilator response to the two different routes of administration were similar. Noradrenaline administered directly into the internal carotid artery, however, caused a reverse effect, and cortical vasoconstriction occurred. The failure to observe this effect following pretreatment with phenoxybenzamine suggests that the vasoconstriction was α-adrenoceptor mediated, and is in agreement with the results of Oberdoster, Lang & Zimmer (1973).

The entirely different effects of noradrenaline given into the internal and common carotid arteries in the present study suggests that the carotid sinus baroreceptors and carotid body chemoreceptors may be involved in the cerebrovascular response to the drug. Hence, noradrenaline given into the common carotid artery reaches the carotid receptors, whereas that given directly into the internal carotid artery does not. Ponte & Purves (1974) have shown that stimulation of carotid body chemoreceptors with hypercapnia or hypoxia can reflexly affect cerebral blood flow. Joels & White (1968) showed that locally applied noradrenaline can increase carotid body chemoreceptor discharge in the cat. Black *et al.*, (1972) have obatined results in the dog which indicate that noradrenaline may stimulate respiration by an action on the carotid body.

It appears, therefore, that the direct action of noradrenaline on cerebral blood vessels is α-adrenoceptor mediated vasoconstriction. However, noradrenaline administered below the level of the carotid reflexogenic areas causes cerebral vasodilatation.

Administration of noradrenaline intravenously once both vagi and sinus nerves had been cut failed to cause the usual flow increase. Cortical blood flow was initially low and it is therefore difficult to interpret these findings as conclusive evidence of the importance of the afferent nerves in the noradrenaline response. It could be argued for example that the cerebral arteries were in spasm and were incapable of dilating in the normal fashion.

In the cross perfusion experiments the resting values for cerebral blood flow were again low. Under these circumstances there was the added factor of internal carotid artery occlusion. However, the dog has very extensive intra- and extracranial communicating vessels and can in fact easily survive ligation of both common carotid arteries and both vertebral arteries (Hill, 1896; Evans & Samaan, 1936).

The control cerebral blood flow values in the deafferentation and in the cross perfusion experiments were similar. In the cross perfusion experiments when 5% CO_2 in air was administered to the donor dog the cerebral blood flow of the recipient dog increased. These results are in agreement with those of Ponte & Purves (1974). The increase in blood flow occurring at a time when mean arterial blood was relatively constant certainly argues that these vessels were able to dilate. Furthermore, this increase is likely to be due to increased activity in the sinus nerve since section of the sinus nerve at the end of the experiment abolished the increase (3 dogs). The increase in flow that occurred following noradrenaline administration to the donor dog was also likely to be affected by this mechanism.

It has been shown that noradrenaline in the dog causes an increase in chemoreceptor discharge (Joels & White, 1968; Black *et al.*, 1972). When CO_2 and noradrenaline were given simultaneously to the donor dog a greater vasodilation in the recipient cerebral circulation occurred. The effects of both these stimuli together seemed to be additive.

Certainly this increase contrasts with the marked fall in flow found when CO_2 and noradrenaline were given together in the single dog experiments.

These results suggest that intravenous noradrenaline probably causes cerebral vasodilatation by stimulating the peripheral chemoreceptors and that the antagonist effect of 5% CO_2 administration must occur in the intact animal at a site different from the peripheral chemoreceptors.

This work was supported by an M.R.C. grant to one of us (I.M.J.). The authors are indebted to Mr Stephen Hall for excellent technical assistance.

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(Received October 7, 1974,
Revised January 22, 1975)

Br. J. Pharmac. (1975), 54, 145-155

MYOCARDIAL FAILURE WITH ALTERED RESPONSE TO ADRENALINE IN ENDOTOXIN SHOCK

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1 There is a growing concensus that myocardial performance in the early stages of experimental endotoxic and septic shock is relatively normal; however, recent reports have identified an intermediate phase of shock when myocardial dysfunction is clearly apparent.

2 The mechanism of dysfunction has become a subject of intense investigation. A current view is that altered myocardial responsiveness to circulating catecholamines may play an important role in the dysfunction observed after endotoxin administration. The present studies, in which an isolated working heart preparation of the dog was used, were designed to test this hypothesis. This particular experimental preparation was selected to provide an adequate interpretation of results; cardiac output, afterload, and concentrations of adrenaline reaching the coronary vascular bed were controlled in all experiments. Responses to infusions of adrenaline were recorded in the 'steady-state' condition. Control (non-shocked) heart responses to adrenaline were highly reproducible in terms of inotropic, chronotropic and coronary vascular behaviour.

3 Results from the study document myocardial dysfunction within 4-6 h following an LD_{70} endotoxin administration on the basis of increased left ventricular end diastolic pressure (LVEDP), decreased cardiac power and myocardial efficiency, and depressed negative and positive dP/dt parameters.

4 Findings suggest significantly altered responsiveness of the myocardium to infused adrenaline at rates of 1, 2, and 5 μg/min with concentrations between 10 and 1 ng/ml blood. LVEDP was elevated while calculated power and efficiency parameters remained significantly below control values during infusion of adrenaline in endotoxin-treated hearts. Depressions of responsiveness were interpreted to occur on the basis of failure to restore positive and negative dP/dt to normal values and depressed coronary blood flow responses during adrenaline administration. Increases in coronary flow were regularly less in experimental hearts than the controls. Heart rate responses to adrenaline in both failing and non-failing hearts were identical.

5 In conclusion, it is suggested that myocardial contractile and relaxation characteristics and coronary vascular responses to adrenaline infusion are depressed in endotoxin shock during the period of demonstrated myocardial dysfunction. No distinct causal relationships were observed between the altered myocardial responsiveness and pathogenesis of heart dysfunction since myocardial dysfunction and altered responsiveness to adrenaline were generally observed together. Myocardial oedema formation after endotoxin as previously reported by this laboratory may bear a relationship to the depressed negative dP/dt response to adrenaline.

Introduction

Classically described early haemodynamic changes in endotoxin shock consist of progressive decreases in venous return and cardiac output occurring concomitantly with marked systemic hypotension in both canine (Weil, MacLean, Visscher & Spink, 1956; MacLean & Weil, 1956) and subhuman primate species (Hinshaw, Shanbour, Greenfield & Coalson, 1970). However, since early peripheral sequestration of blood is clearly observed to occur, the decrease in cardiac output has been accounted for on the basis of diminished venous return (Weil *et al.*, 1956). There is a growing concensus of opinion that myocardial performance in the early stages of shock (0-3 h) is relatively normal and that depressed cardiac performance *per se* performs an insignificant role in the early reduction of cardiac output (Weil *et al.*, 1956; Goodyer, 1967; Hinshaw, Archer, Greenfield & Guenter, 1971a; Hinshaw, Archer, Greenfield, Guenter & Miller, 1972a; Hinshaw, Greenfield, Owen, Black & Guenter, 1972c; Siegel, Farrell, Goldwyn & Friedman, 1972). It has been proposed by Goodyer (1967) and Hinshaw *et al.* (1971a) and inferred from the work of Siegel *et al.* (1972) that ventricular performance in the initial stage of endotoxin or septic shock is supported by a sympathoadrenal response initiated subsequent to the development of systemic hypotension, which is consistent with the findings of others (Nykiel & Glaviano, 1961; Hökfelt, Bygdeman & Sekkenes, 1962; Cavanagh, Rao, Sutton, Bhagat & Bachmann, 1970; Hall & Hodge, 1971). Increased neurohumoral activity associated with the early compensatory period of shock should provide substantial cardiovascular support toward the maintenance of normal haemodynamics in the face of diminished venous return.

Recent reports have identified a subsequent phase of shock, following the early compensatory period, when myocardial performance begins to falter or fail altogether (Solis & Downing, 1966; Goodyer, 1967; Siegel, Greenspan & Del Guercio, 1967; Cavanagh *et al.*, 1970; Bell & Thal, 1970; Cann, Stevenson, Fiallos & Thal, 1972; Hinshaw *et al.*, 1972c; Hinshaw, Archer, Black, Greenfield & Guenter, 1973; Parratt, 1973; Nishijima, Weil, Shubin & Cavanilles, 1973; Hinshaw, Archer, Black, Elkins, Brown & Greenfield, 1974a; Parratt & Winslow, 1974; Greenfield, Jackson, Elkins, Coalson & Hinshaw, 1974) in both animal endotoxic studies and clinical septic shock. The observed myocardial dysfunction in not merely a preterminal event and is not necessarily associated with systemic hypotension, acidemia, or depressed oxygen delivery (Hinshaw *et al.*, 1972c, 1973; Hinshaw, Archer, Spitzer, Black, Peyton & Greenfield, 1974b), and does not appear to be due to the release of a myocardial depressant factor (Hinshaw, Greenfield, Owen, Archer & Guenter, 1972b; Hinshaw *et al.*, 1974a). One possible cause of the dysfunction may be a depressed response of the myocardium to circulating catecholamines following the period of increased sympathetic activity, and this potential mechanism is the subject of the present study. Recent research, supportive of this view, is that of Cavanagh *et al.* (1970), Bhagat, Cavanagh, Merrild, Rana & Rao (1970), and Parratt (1973), who reported depressed inotropic, chronotropic or altered coronary blood flow responses in various species of animals subjected to endotoxin shock. Relatively depressed myocardial responsiveness to neurally-induced sympathetic stimulation in shock has been reported by Glaviano & Klouda (1965) and Geocaris, Quebbeman, Dewoskin & Moss (1973). On the other hand, excessive sympathetic stimulation of the myocardium in rabbits administered endotoxin may elicit subendocardial haemorrhage and necrosis of myocardial fibres (Palmerio, Ming, Frank & Fine, 1962). Results from the present study partially support the earlier observations by Cavanagh *et al.* (1970), Bhagat *et al.* (1970), and Parratt (1973) once myocardial failure is clearly demonstrated, but provide no evidence for their initiating roles in the pathogenesis of myocardial dysfunction.

Methods

Experiments were carried out on nineteen isolated working canine hearts supported with arterial blood from a heparinized support dog, prepared as previously described (Hinshaw *et al.*, 1971a, 1973). The donor dog was anaesthetized, and the chest opened by median sternotomy after the animal was placed on a constant volume respirator. The azygos vein and subclavian artery were ligated and divided. Ligatures were loosely placed around the thoracic aorta distal to the subclavian artery, the brachiocephalic artery, and superior and inferior venae cavae. The pericardial sac was opened along its ventral surface and the animal was injected with heparin (3-5 mg/kg). The vagi were then cut in the neck and the brachiocephalic artery was cannulated with a Tygon tube elevated to a height of 100-125 cm above the heart level. The superior vena cava was cannulated with a blood-filled plastic tube led through a roller blood pump prepared to draw blood from the aorta of the support dog. Blood was allowed to flow through the brachiocephalic cannula and to fill the Tygon tube. The aorta of the isolated heart was then tied distal to the origin of the brachiocephalic artery, the superior vena caval inflow from the pump was started at about 120 ml/min, and the inferior vena cava was immediately ligated. Blood from the aortic outflow of the isolated heart was collected in a plastic reservoir placed within a heated water bath, and returned to the dog at a flow rate equal to the superior vena caval inflow. Heart, lungs and adjoining tissue were then removed from the chest and supported by the trachea in the external system with adequately provided coronary pressure and blood flow. The lungs of the isolated heart were ventilated, while the support animal was respired spontaneously. Hearts were removed without interruption of blood flow from adult donor animals weighing between 4.5 and 8.0 kg and continuously supported by pump-delivered blood from adult support dogs weighing between 22 and 32 kg.

The right heart was then bypassed after first passing a saline-filled plastic tube into the right ventricle via the atrium, and then cannulating the pulmonary artery from a 'T'-connector previously secured to the superior vena caval inflow tubing. The cannulation of the pulmonary artery required only a few seconds during which time the coronary vessels were retrogradely perfused with blood by hydrostatic pressure from the aortic outflow tubing. Left ventricular pressure was measured simultaneously for end diastolic pressure (0-40 mmHg) and systolic pressure (0-200 mmHg) by means of separate Statham pressure transducers attached by a 'Y'-connector to a plastic cannula inserted through a purse-string suture in the apex of the left ventricle.

Coronary venous blood was collected from the right ventricular drain into a plastic reservoir and returned together with brachiocephalic outflow to the support dog via a second pump. Cardiac output was taken as the sum of aortic outflow and coronary flow, both measured with a cylinder and stopwatch. Aortic pressure, left ventricular pressures, cardiac contractility and ECG of the isolated heart, and systemic pressure of the support animal, were monitored continuously on a Sanborn recorder. The first derivative of the left ventricular pressure, $\mathrm{d}P/\mathrm{d}t_{max}$, was continuously recorded by means of a resistance-capacitance differentiating network. Mean aortic pressure was controlled in the isolated heart preparation by adjustment of a screw clamp on the aortic outflow. Cardiac output was maintained at 76 ml kg^{-1} min^{-1} by adjustment of pump speed supplying the pulmonary artery.

Following an equilibrium period of approximately 30 min when a steady-state condition was reached, samples of coronary arterial and venous blood were collected and analyzed for blood gases, with an Instrumentation Laboratories blood gas analyzer. Oxygen content in coronary arterial and venous blood was measured by a Van Slyke manometric blood gas analyzer. Blood temperatures of isolated heart and support dog were measured continuously with thermistor probes and held constant by use of heating pads and water bath temperature regulation. Methods used for experimental procedures and statistical treatment of data have been described previously (Hinshaw *et al.*, 1974a, 1974b).

All isolated hearts received periodic and variable dose infusions of adrenaline (Parke, Davis & Co.) introduced into the left ventricle via a polyethylene catheter whose distal tip was placed in the chamber of the left atrium with the proximal end attached to a motor-driven syringe. Following measurement of control myocardial parameters without adrenaline, dose-response curves were carried out at regular intervals, all values being recorded during steady-state conditions. Adrenaline infusion rates were varied between 1, 2 and 5 μg/minute. These rates of infusion resulted in adrenaline blood concentrations of 1-10 ng/ml, based on the dilution of the infused drug with the cardiac output (average, 500 ml/min). Adrenaline was infused at afterloads of 50 mmHg and a cardiac output of 76 ml min^{-1} kg^{-1}, based on the weight of the heart donor animal. Afterloads were maintained at 100 mmHg between adrenaline infusions.

A control group of six hearts, receiving no endotoxin, was monitored at 100 and 50 mmHg afterloads for 4-5 h after the beginning of the heart perfusion period when pressures and flows became stabilized. Inotropic, chronotropic and coronary vascular responses to adrenaline infusions administered to control (non-shocked) hearts demonstrated a high degree of reproducibility during the 4-5 h period in the isolated state.

The experimental group consisted of thirteen isolated hearts and support animals receiving an LD_{70} dose of *Escherichia coli* endotoxin, 1.5 mg/kg. Endotoxin was obtained from Difco Laboratories, Detroit, Michigan (Lipopolysaccharide B, *E. coli* 0127 : B8, Boivin procedure with TCA extraction). The degree of lethality was previously determined on a group of 30-40 animals.

Seven anaesthetized support dogs and seven heart donor animals administered endotoxin were observed approximately 1-2 h before isolation of the left ventricle and its removal to the perfusion apparatus. In addition, six hearts and their respective support dogs were given endotoxin following transfer, equilibration and sampling for control values. As in the control group, samples were taken at 100 and at 50 mmHg afterload, and adrenaline responses were reported at 50 mmHg. Experimental and control isolated heart preparations were observed alike during the 4-5 h period in the isolated state.

Results

Results are divided into three categories: (a) those describing the effects of endotoxin on the support animal supplying blood to the isolated heart; (b) those documenting the development of myocardial dysfunction in the isolated heart during the 6 h post-endotoxin period; and (c) those illustrating changes in myocardial function (LVEDP, + and $-\mathrm{d}P/\mathrm{d}t_{max}$), work performance (power), heart rate, and myocardial

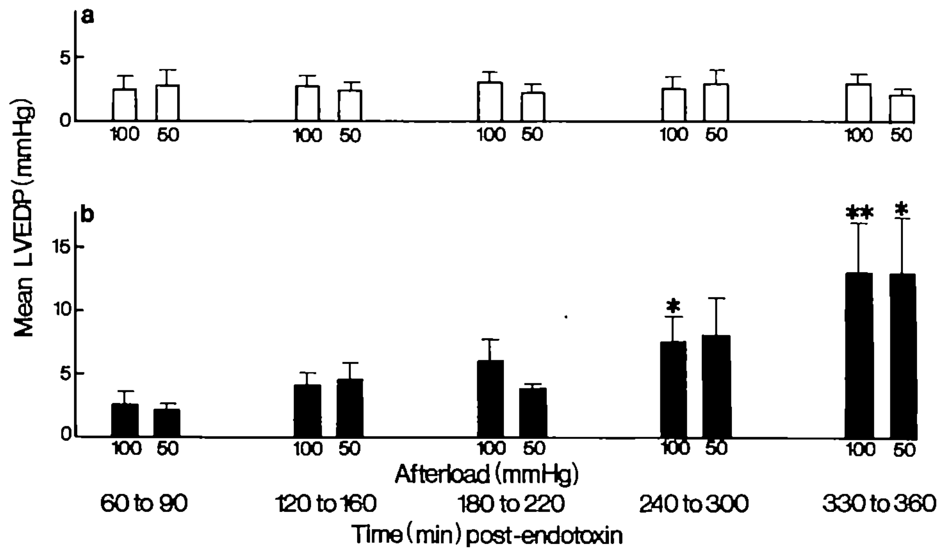

Figure 1 Changes in left ventricular end diastolic pressure (LVEDP) following administration of endotoxin (mean ± s.e., afterloads of 100 and 50 mmHg). Isolated canine hearts, (a) 6 control (no endotoxin), and (b) 13 experimental preparations, 0-6 h post-endotoxin. * $P < 0.05$; ** $P < 0.01$.

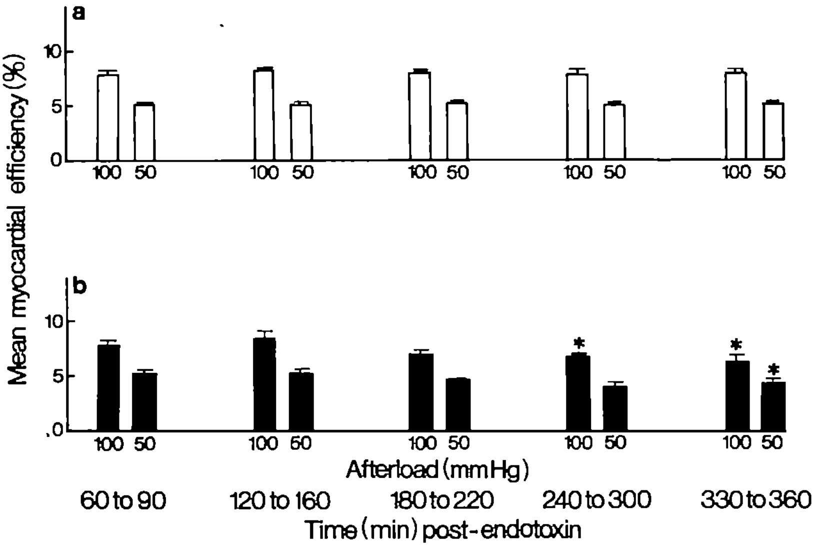

Figure 2 Effects of endotoxin on myocardial efficiency (mean ± s.e., afterloads of 100 and 50 mmHg). (a) 6 control (no endotoxin) and (b) 13 experimental heart preparations studied 0-6 h post-endotoxin. * $P < 0.05$; ** $P < 0.01$.

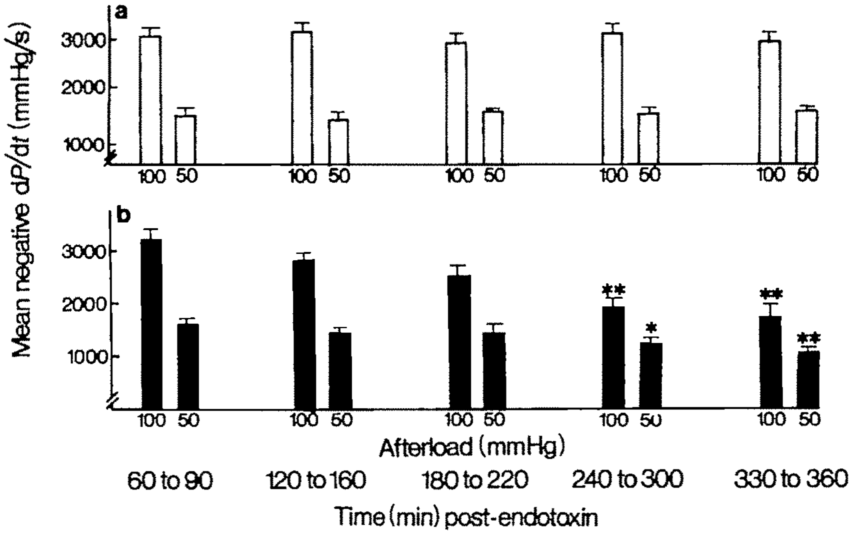

Figure 3 Decreases in mean negative d*P*/d*t* (—d*P*/d*t*) in (a) 6 isolated control (no endotoxin) hearts and (b) 13 experimental hearts studied 0-6 h post-endotoxin (mean ± s.e.). $^*P < 0.05$; $^{**}P < 0.01$.

haemodynamics (coronary blood flow) in response to graded doses of infused adrenaline.

Table 1 describes responses of support animals used in the current series of experiments to endotoxin administration and documents the sustained presence of systemic hypotension, in contrast to the normally maintained systemic pressures in control dogs not receiving endotoxin.

Figures 1, 2 and 3 and Table 2 illustrate the development of detrimental myocardial performance characteristics documenting cardiac dysfunction after endotoxin in the current series of heart studies. No statistically significant differences between experimental and control hearts were seen in the observed parameters until 4 h after endotoxin injection. Data from thirteen experimental heart preparations demonstrate significant elevations in left ventricular end diastolic pressure (LVEDP) ($P < 0.05$, Figure 1), depressions in myocardial efficiency ($P < 0.05$, Figure 2), negative $\mathrm{d}P/\mathrm{d}t_{max}$ ($P < 0.05$, Figure 3), and positive $\mathrm{d}P/\mathrm{d}t_{max}$ ($P < 0.05$, Table 2) at afterloads of 100 and 50 mmHg between 4 and 6 h post-endotoxin. The most outstanding readily

Table 1 Systemic arterial pressure response* of heart-support animals following LD_{70} endotoxin administration

Time post-endotoxin (min)	*Experimental group (n = 13)*	P	*Control group (n = 6)*
+60 to	85(7)	< 0.01	143(5)
+90	85(5)	< 0.01	133(5)
+120 to	80(8)	< 0.01	138(8)
+160	81(6)	< 0.01	138(8)
+180 to	104(9)	< 0.01	138(6)
+220	109(7)	< 0.05	137(7)
+240 to	90(8)	< 0.01	143(5)
+300	91(6)	< 0.01	133(5)
+330 to	83(10)	< 0.01	138(6)
+360	90(6)	< 0.01	137(7)

* mmHg; mean with (s.e. mean)

observable changes signalling the onset of myocardial dysfunction are increases in LVEDP to values exceeding 10 mmHg at low afterloads and progressively developing depressions of negative d*P*/d*t*. The possible significance of the latter is discussed in a recent report (Hinshaw *et al.*, 1974b).

Table 3 summarizes steady-state changes in myocardial O_2 uptake following endotoxin administration. In general, mean O_2 uptake values are lower in the experimental hearts compared with the controls not receiving endotoxin, and are significantly decreased within 4-5 h at afterloads of 50 and 100 mmHg, and from 5-6 h post-endotoxin at 50 mmHg ($P < 0.05$). Myocardial arterial Po_2 was significantly decreased ($P < 0.05$) from 2-5 h after endotoxin, maximum decreases ranging from an initial value of 112(±6) to 98(±3) mmHg at 5 hours.

Figures 4-6 summarize steady-state responses of the isolated heart preparation to different concentrations of adrenaline at various times following endotoxin during controlled afterloads and cardiac outputs. Control hearts not receiving endotoxin are similarly infused with adrenaline at the same time intervals. Adrenaline is infused into the left atrium during a fixed cardiac output (76 ml min^{-1} kg^{-1}, in order to avoid problems of variable dilution of adrenaline in the pulmonary bed and to insure delivery of identical adrenaline concentrations to the coronary bed. Afterloads were fixed at 50 mmHg in order to insure identical coronary perfusion pressure characteristics. All responses shown are steady-state values, ordinarily

Table 2 Changes in left ventricular positive d*P*/d*t** (+d*P*/d*t*) following endotoxin administration

Time post-endotoxin (min)	*Afterload (mmHg)*	*Experimental group* (n = *13)*	P	*Control group* (n = *6)*
+60 to	100	2281(125)		2120(115)
+90	50	1295(45)		1200(75)
+120 to	100	2030(135)		2055(85)
+160	50	1145(65)		1110(40)
+180 to	100	2040(150)		2110(90)
+220	50	1160(50)		1185(65)
+240 to	100	1715(135)	< 0.05	2120(115)
+300	50	1075(45)		1200(75)
+330 to	100	1675(80)	< 0.01	2110(90)
+360	50	945(90)	< 0.05	1185(65)

* mmHg/s; mean (± s.e.) (76 ml min^{-1} kg^{-1} cardiac output at all afterloads)

Table 3 Changes in O_2 uptake* following endotoxin administration

Time post-endotoxin (min)	*Afterload (mmHg)*	*Experimental group* (n = *13)*	P	*Control group* (n = *6)*
+60 to	100	11.3(1.0)		12.0(0.6)
+90	50	8.5(0.7)		9.0(0.5)
+120 to	100	9.8(1.6)		11.3(0.4)
+160	50	7.6(1.2)		9.0(0.4)
+180 to	100	11.9(0.8)		11.9(0.5)
+220	50	9.1(0.7)		8.9(0.6)
+240 to	100	10.1(0.6)	< 0.05	12.0(0.6)
+300	50	7.6(0.4)	< 0.05	9.0(0.5)
+330 to	100	10.7(0.5)		11.9(0.5)
+360	50	7.0(0.7)	< 0.05	8.9(0.6)

* ml min^{-1} per 100 g left ventricle, mean (± s.e.)

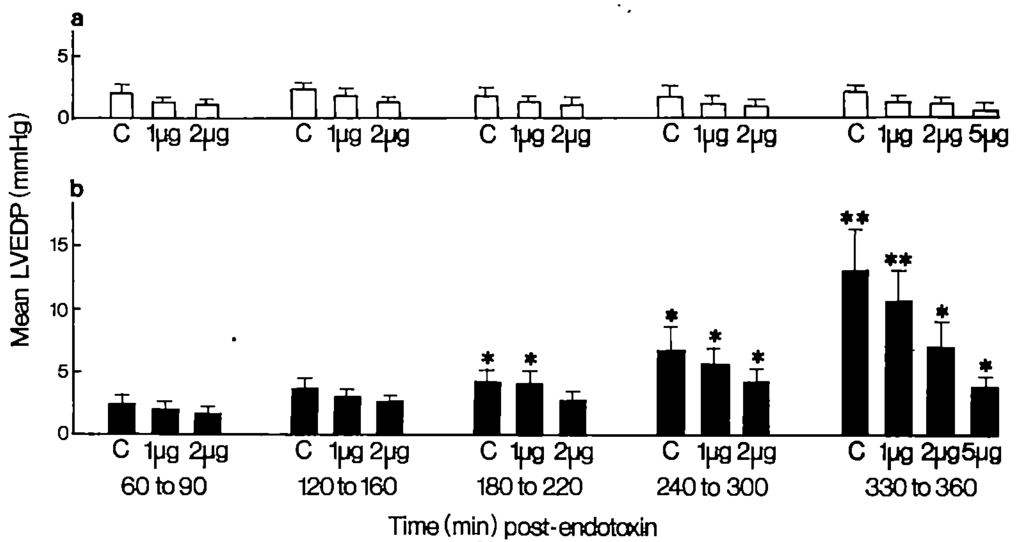

Figure 4 Mean steady-state values (± s.e.) of left ventricular end diastolic pressure (LVEDP) during adrenaline infusion into left atrium of isolated heart after endotoxin (LD_{70}). (a) Six control (no endotoxin) and (b) 13 experimental hearts studied 0-6 h post-endotoxin. * $P < 0.05$; ** $P < 0.01$.

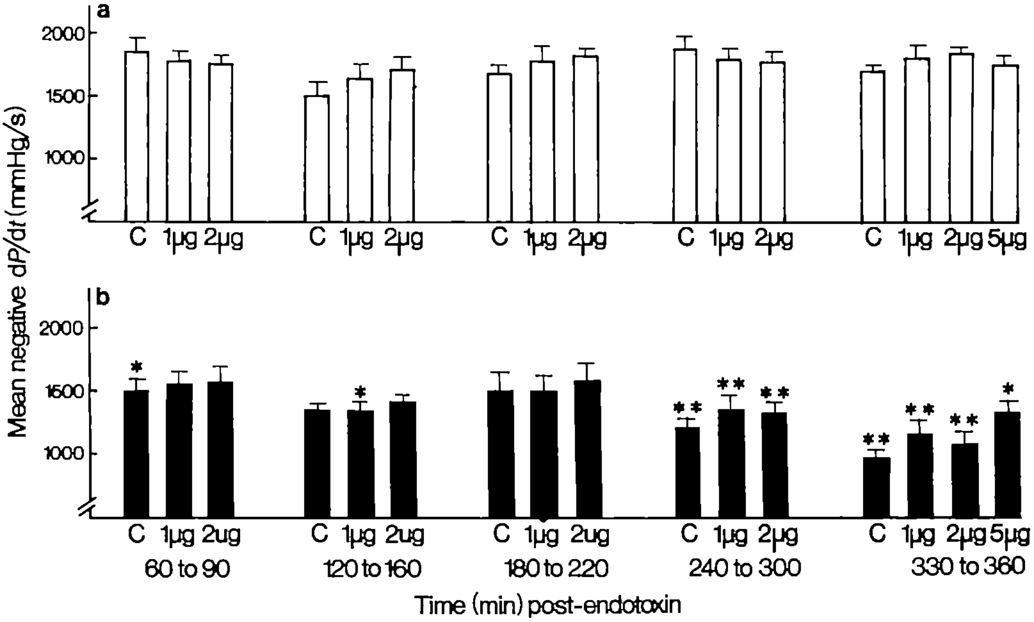

Figure 5 Mean steady-state values (± s.e.) of left ventricular negative dP/dt (—dP/dt) during adrenaline infusion into left atrium, 0-6 h post-endotoxin. (a) Six control (no endotoxin) and (b) 13 experimental hearts. * $P < 0.05$; ** $P < 0.01$.

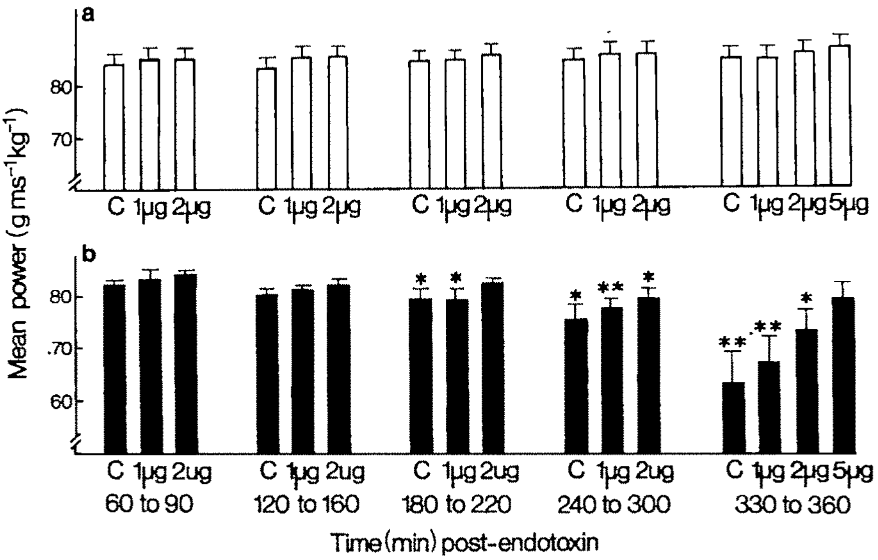

Figure 6 Mean steady-state values of left ventricular power (g.m s^{-1} kg^{-1}) during left atrial infusions of adrenaline 0-6 h after endotoxin. (a) Six control (no endotoxin) and (b) 13 experimental hearts (mean ± s.e.). * $P < 0.05$; ** $P < 0.01$.

achieved within 2-4 min of adrenaline infusion. The upper panel of Figure 4 shows mean LVEDP responses of six control hearts (no endotoxin) to adrenaline during a 4-5 h period. Results show that successive responses to 1 and 2 μg/min infusions of adrenaline in control hearts are reproducible and indistinguishable in magnitude at the same doses. On the other hand, the lower frame of Figure 4 shows that values of LVEDP in endotoxin-treated hearts are considerably higher, and significant differences occur between control and experimental (endotoxin) preparations from 3-6 h after endotoxin before and during adrenaline administration ($P < 0.05$). LVEDP values in failing hearts decreased during infusion of adrenaline but remained consistently higher than those from hearts not subjected to endotoxin at 1, 2 and 5 μg/min infusions ($P < 0.05$). Probability values shown in Figure 4 refer to differences between control (no endotoxin) and experimental (endotoxin) groups in response to adrenaline infusion at the same time interval.

Figure 5 shows negative $\mathrm{d}P/\mathrm{d}t_{max}$ values in response to adrenaline infusion, as administered in the previous figure. Results from dysfunctioning hearts show decreases in negative $\mathrm{d}P/\mathrm{d}t_{max}$ ($P < 0.05$) during the later phase of shock (4-6 h), which were little changed by the administration of adrenaline. Decreases in positive $\mathrm{d}P/\mathrm{d}t_{max}$ were evident at 6 h post-endotoxin during adrenaline infusions of 1 and 2 μg/min ($P < 0.05$). Depressions in myocardial power are observable within 3 h after endotoxin and decline progressively ($P < 0.05$) up to the sixth hour (Figure 6). Infusions of 1 and 2 μg/min adrenaline failed to restore myocardial power to normal values ($P < 0.05$). At termination of the experiments, 5 μg/min adrenaline was infused and restored power to control values.

Coronary blood flow after endotoxin was higher during the early phase of shock than in the control non-shocked heart, reaching a peak of 96 ml min^{-1} per 100 g left ventricle (control, 78 ml/min) at 2 h post-endotoxin, thereafter declining steadily to 72 ml/min (control, 73 ml/min) within 6 hours. Mean increases in coronary blood flow in response to 1-2 μg/min infusions of adrenaline were regularly less in experimental hearts than the controls ($P < 0.05$), and this is particularly evident by 6 h of shock during an infusion of 5 μg/min adrenaline ($P < 0.05$). Results indicate that heart rate changes after endotoxin were no different from control non-shocked preparations. Heart rate responses to adrenaline infusions of 1, 2 and 5 μg/min were identical in both control and experimental hearts, showing notable increases in both series. At termination of the studies, during 5 μg/min infusions, rates of experimental hearts increased from 144 to 170 while controls were elevated similarly from 143 to 168 beats/minute.

Discussion

The primary initial factors previously reported to be instrumental in lowering cardiac output during the first 1-2 h of endotoxin shock are peripheral vascular pooling, extravasation of plasma, and the resultant effect of decreased venous return (Weil *et al.*, 1956; MacLean & Weil, 1956; Hinshaw, Shanbour, Greenfield & Coalson, 1970). Myocardial performance in the early phase of shock appears to be relatively normal (Goodyer, 1967; Hinshaw *et al.*, 1971a, 1972a; Hinshaw, Greenfield, Archer & Guenter, 1971b), and the subsequent induction of systemic hypotension elicits sympathoadrenal stimulation of the heart, augmenting its function (Nykiel & Glaviano, 1961; Hökfelt *et al.*, 1962; Cavanagh *et al.*, 1970; Hall & Hodge, 1971; Hinshaw *et al.*, 1971a; Siegel *et al.*, 1972; Geocaris *et al.*, 1973).

Substantial evidence has been produced for a prominent role of the myocardium in the ultimate development of irreversible endotoxin (septic) shock in a wide spectrum of animal species and in man himself (Hinshaw, 1974). The purpose of the present study was to evaluate adrenergically related mechanisms which might account for the eventual depression of myocardial function occurring from 2-6 h after administration of endotoxin or live *E. coli* organisms, as reported by Goodyer (1967), Cavanagh *et al.* (1970), Hinshaw *et al.* (1972c, 1973), Parratt (1973), Parratt & Winslow (1974), and Greenfield, Jackson, Elkins, Coalson & Hinshaw (1974). It was hoped that similar mechanisms to those observed in certain mammalian species might also apply to an understanding of the development of heart dysfunction in clinical septic shock (Siegel *et al.*, 1967, 1972; Bell & Thal, 1970; Cann *et al.*, 1972; Nishijima *et al.*, 1973).

One possible cause of cardiac dysfunction as studied in the present investigation was an altered myocardial responsiveness to circulating catecholamines which could presumably reduce cardiac output. Substantial evidence for this possibility has been presented by others (Cavanagh *et al.*, 1970; Bhagat *et al.*, 1970; Parratt, 1973). Limitations of these earlier reports were that either only portions of the heart were used in the tests for responsiveness, excluding the left ventricle itself, or that provisions were lacking to insure a constant delivery of catecholamine to the myocardial coronary circulation after the induction of shock. The methodology of the present study, while fixing cardiac inflow (cardiac output) and afterload at constant values (76 ml min^{-1} kg^{-1} and 50 mmHg, respectively) in the isolated working heart preparation during constant infusion rates of adrenaline into the left ventricle, rather than peripheral vein, assured better controlling features of the experiments for adequate interpretation of the data. Another advantage of the present preparation was that responses of control (non-shocked) hearts to adrenaline were found to be highly reproducible during a 4-5 h period of observation. Responses of experimental hearts, on the other hand, were notably different.

Results from the present study document myocardial dysfunction within 4-6 h after an LD_{70} endotoxin administration, as evidenced by statistically significant elevations of LVEDP and depressions of myocardial efficiency, power, and negative and positive dP/dt_{max} at afterloads of 50 and 100 mmHg. Intact support dogs, exchanging blood with the isolated heart and also receiving endotoxin, were markedly hypotensive during the 6 h observation period. The notable decrease in negative dP/dt_{max} values is considered to be associated with diminished effectiveness of cardiac filling during diastole (Hinshaw *et al.*, 1974b).

Findings from the present study suggest significantly altered responsiveness of certain myocardial performance and haemodynamic parameters to adrenaline infusions in hearts receiving endotoxin. LVEDP values in failing hearts decreased during administration of adrenaline but remained consistently higher than those in control hearts, even when high concentrations of adrenaline (5 μg/min) were used to challenge the myocardium. Decreases in both positive and negative dP/dt_{max} in dysfunctioning hearts could not be restored to normal by adrenaline infusion, and this was particularly noted by the observed negative dP/dt_{max} responses, presumably reflecting impaired cardiac filling characteristics. On the other hand, large doses of adrenaline (5 μg/min) essentially restored cardiac power calculations to normal in failing hearts, which was apparently due to improved cardiac contractility characteristics. Mean increases in coronary blood flow in response to adrenaline infusion were regularly less in experimental hearts than the controls, and this observation may bear a causal relationship to the development of heart dysfunction after endotoxin. This was particularly evident within 5-6 h of shock during an infusion of 5 μg/min adrenaline ($P < 0.05$). Heart rate, in contrast, responded similarly to adrenaline in both failing and non-failing (control) preparations. These findings suggest relatively depressed responsiveness of the canine heart model to adrenaline administration during the development of myocardial failure. Depressed myocardial efficiency and decreased inotropic and coronary blood flow responses to adrenaline were regularly observed while chronotropic characteristics were not modified in the shocked state. These results are in agreement with Parratt's study (1973) in endotoxin-treated cats, except that a decreased chronotropic responsiveness to adrenaline was also reported. Bhagat *et al.* (1970) observed a reduced sensitivity of isolated guinea-pig atria to noradrenaline after endotoxin which reached a maximum depression within 6-18 h after endotoxin administration. Cavanagh *et al.* (1970) described a depressed responsiveness of the isolated baboon atrium to noradrenaline within 4 h after endotoxin. These guinea-pig and baboon atrial responses bear distinct similarities to our canine heart results with adrenaline, both as to type of depression and time required for its appearance after endotoxin.

Reasons for reduced responsiveness to adrenaline or noradrenaline are not known. In an interesting recent investigation, Drucker, Pindyck, Brown, Elwyn & Shoemaker (1974) pointed out that hypertonic glucose infusion in patients greatly enhanced the myocardial inotropic effect of administered glucagon. The augmented myocardial response to glucagon in the presence of adequate glucose included increased cardiac output and left ventricular stroke work. In a recent study (Hinshaw, Peyton, Archer, Black, Coalson & Greenfield, 1974c), a significantly increased survival rate was observed when hypoglycemia elicited by endotoxin in dogs was prevented by infusing hypertonic glucose in amounts sufficient to maintain arterial blood glucose concentrations constant. In view of both this study and the findings of Drucker *et al.* (1974), it might be surmised that myocardial inotropic responses to catecholamines in shock could be improved in the presence of adequate substrate delivery to the myocardium.

Another suggested mechanism for reduced myocardial responsiveness to adrenaline is that endotoxin may inhibit adenosine 5′-triphosphate (ATP)-dependent calcium uptake by the sarcoplasmic reticulum, which could depress the contraction process (Hess & Briggs, 1971). Shinebourne, Hess, White & Hamer (1969) reported that the effect of noradrenaline on myocardial contractility is directly related to the increased rate of calcium uptake by the sarcoplasmic reticulum. A similar linkage may have explained the increased inotropic responses of hearts administered endotoxin and treated with digoxin (Hinshaw *et al.*, 1973). Finally, decreases in pH have been implicated in explaining altered myocardial responsiveness to catecholamine stimulation (Thrower, Darby, Aldinger, Tenney & Westbrook, 1959; Darby, Aldinger, Gadsen & Thrower, 1960). In the present study, mean arterial pH values in both control and experimental groups ranged between 7.35 and 7.45, and there was no apparent correlation between responsiveness to adrenaline and pH in individual experiments.

The precise role of the sympathoadrenal system in shock is difficult to interpret. Depression of myocardial responsiveness to circulating catecholamines and sympathetic stimuli have been implicated in explaining decreased heart function in shock. On the other hand, excessive sympathoadrenal stimuli are known to elicit severe myocardial tissue damage (Palmerio *et al.*, 1962, Lundsgaard-Hansen, Meyer, Riedwyl & Zierrot, 1969). The relative adverse contributions of these influences on the pathogenesis of myocardial dysfunction in shock remain to be elucidated.

This research was supported by the USPHS NIH grant HL 15037, U.S. Navy Contract N00014-68-A-0496, the Oklahoma Heart Association, and the Veterans Administration Hospital. Appreciation is expressed to Beverly Beller, Jeanette Glasgow and R.T. Brantley for technical assistance.

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*(Received October 15, 1974.
Revised December 24, 1974)*

Br. J. Pharmac. (1975), 54, 157-162

ROLE OF CATECHOLAMINES IN THE CENTRAL MECHANISM OF EMETIC RESPONSE INDUCED BY PERUVOSIDE AND OUABAIN IN CATS

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1 Peruvoside, (a glycoside obtained from the plant, *Thevetia neriifolia Juss*) and ouabain produce emesis in cats. Vomiting is not produced by these drugs in animals pretreated with catecholamine depleting drugs like reserpine, tetrabenazine or syrosingopine. Chloropromazine hydrochloride, mepyramine maleate, or BOL-148 administered intravenously or intracerebroventricularly do not afford protection.

2 Phenoxybenzamine produces partial protection against peruvoside-induced emesis.

3 Haloperidol (1 mg/kg i.v.) prevents vomiting induced by peruvoside or ouabain. Intracerebroventricularly administered haloperidol is ineffective.

4 Cats pretreated with SKF-525-A, are not protected by haloperidol. Animals pretreated with phenobarbitone in a dose of 25 mg/kg for a week were protected by haloperidol, 250 μg/kg i.e. one quarter of the effective antiemetic dose in normal cats.

5 It is postulated that catecholamines are involved in the mechanism of vomiting induced by cardiac gycosides. Further, a metabolite of haloperidol seems to be responsible for its effective antiemetic action.

Introduction

The chemoreceptor trigger zone in the area postrema has been shown to be the central site of emetic response induced by a number of drugs (Borison & Brizzee, 1951; Wang & Borison, 1952). This area is rich in catecholamines (Vogt, 1954). Borison (1959) and Feldberg & Sherwood (1954) have shown that intracerebroventricular administration of catecholamines induced emesis in dogs and cats. Cahen (1964) reported that 5-hydroxytrytophan-induced emesis in cats was brought about through the release of catecholamines. Koppanyi & Cowan (1962) reported catecholamines as possible stimulants of chemoreceptor mechanisms. Peng (1963) has shown that dihydroxyphenylalanine (DOPA) induced emesis in dogs. We have investigated the role of catecholamines in the mechanism of the emetic response induced by ouabain and peruvoside. In our investigation, we found that haloperidol possessed a potent antiemetic activity and it was decided to investigate further the mechanism of this action of haloperidol.

Methods

Cats of either sex weighing between 2-4 kg were used. Various doses of peruvoside or ouabain were administered via the saphanous vein by a rapid injection in volumes not exceeding 3 ml.

A cannula was placed in the lateral cerebral ventricle according to a method described by Feldberg & Sherwood (1954). The position of the cannula was verified at the end of each experiment. All emetic tests were performed shortly after feeding. Expulsion of gastric contents was taken as a positive response. The functional integrity of the vomiting mechanism was confirmed after each experiment by oral administration of copper sulphate (20 mg in 50 ml of water) (Wang & Borison, 1952).

In order to explore the role of catecholamines, various catecholamine depleting drugs or antagonists were used. The doses, route and the time of challenge are given in Table 1.

To assess the role of microsomal enzymes, a group of 20 cats, received SKF-525-A (βdiethylaminoethyl diphenylpropyl acetate hydrochloride) in a dose of 75 mg/kg intraperitoneally and the animals were challenged with peruvoside or ouabain after 6 and 24 hours.

Sodium phenobarbitone in a dose of 25 mg/kg was administered intraperitoneally daily, for eight days in 18 cats and the animals were challenged with peruvoside or ouabain.

Drugs

SKF-525-A, was supplied by Smith Kline and French Laboratory, Philadelphia; reserpine and syrosingopine were supplied by Ciba Research Center, India. Tetrabenazine, was supplied by Roche Pharmaceuticals India, haloperidol and pimozide were gifts from Janssen Pharmaceuticals, Belgium. Peruvoside was supplied by the Director, Indian Council of Medical Research, New Delhi, India. Other drugs were obtained from local commercial firms.

Results

Emetic response in cats

The results of administration of peruvoside and ouabain are presented in Table 2. In cats the maximum effective emetic dose of peruvoside was 125 μg/kg given intravenously. The latency of vomiting by the intravenous route was 3-8 min (average 5.5 minutes). A dose of 125 μg/kg intravenously was lethal in 4 out of 20 cats, but the vomiting was not the terminal event. Since this was the most effective dose, it was used in all subsequent experiments. The most effective emetic dose of ouabain was found to be 60 μg/kg intravenously and this was used for comparison.

All cats which vomited had anorexia, lasting for 2-5 days.

Emetic response in cats treated with reserpine, tetrabenazine and syrosingopine

Table 3 illustrates the effect of these catecholamine-depleting drugs, given in the doses shown in Table 1, on emetic response induced by peruvoside or ouabain It can be seen that chronic reserpine-treatment or intracerebroventricular reserpine abolished peruvoside as well as ouabain-induced emesis. The cats were sedated. Cats treated with tetrabenazine (50 mg/kg i.v.) or syrosingopine (10 mg/kg i.v.) did not vomit after administration of peruvoside or ouabain. None of the animals in these groups showed marked sedation. All animals who failed to vomit after peruvoside or ouabain vomited after administration of copper sulphate orally, with a latency of 12-20 min (average 16 minutes).

Table 1 Schedule of treatment of cats challenged with peruvoside or ouabain.

Treatment	*Dose and Route*	*No. of cats*	*Time of challenge*
Reserpine	*1st day* 2 mg/kg i.m. *2nd day* 0.5 mg/kg i.m. *3rd day* 1 h prior to challenge, 0.3 mg/kg i.m.	12	3rd day
Reserpine	500 μg i.c.v. in divided doses	6	4 h
Syrosingopine	10 mg/kg i.v.	12	24 h
Tetrabenazine	50 mg/kg i.v.	12	4 h
Phenoxybenzamine	5 mg/kg i.v.	15	0.5 h
	2 mg i.c.v. in divided doses	11	1 h
Chlorpromazine	2.5 mg/kg i.v.	12	1 h
	750 μg i.c.v. in divided doses	6	0.5 h
Mepyramine maleate	10 mg/kg i.m.	12	1 h
	3 mg i.c.v. in divided doses	6	40 min
BOL–148	50 μg/kg i.v.	12	0.5 h
	20 μg i.c.v.	6	0.5 h
Haloperidol	1 mg/kg i.v.	20	1 h
	250 μg/kg i.v.	28	1 h
	200 μg i.c.v. in divided doses	6	0.5 h
Pimozide	1 mg/kg i.v.	3	24 h

i.v. = intravenous; i.c.v. = intracerebroventricular; i.m. = intramuscular.

Effect of mepyramine maleate, BOL-148, chlorpromazine hydrochloride and phenoxybenzamine

Mepyramine maleate, BOL-148, chlorpromazine hydrochloride intravenously or intracerebroventricularly prior to emetic challenge did not prevent emesis (Table 4).

Phenoxybenzamine afforded protection in 50% of animals challenged with peruvoside.

Antiemetic action of haloperidol and pimozide

Haloperidol given in a dose of 1 mg/kg intravenously, 1 h before the emetic challenge antagonized the action of peruvoside and ouabain. All such cats rendered refractory to emetic action of peruvoside or ouabain by haloperidol, vomited after oral administration of copper sulphate. The results are presented in Table 5.

Haloperidol, 200 µg introduced into the lateral ventricle 30 min before peruvoside or ouabain were administered intravenously, failed to protect the animals against vomiting induced by these drugs, although the drug produced a moderate degree of sedation. Intracerebroventricular haloperidol itself produced vomiting in all the cats

Table 2 Emetic response after intravenous administration of peruvoside and ouabain in cats.

Drug	*Route*	*Dose (µg/kg)*	*No. of cats vomited/tested*	*Latency in min (range)*
Peruvoside	i.v.	50	0/5	–
		75	2/5	20-69
		100	3/5	20-40
		125	20/20*	3-8
		150	5/5**	3-6
Ouabain	i.v.	60	15/15	8-22

i.v. = intravenous; * = four animals died; ** = all animals died.

Table 3 Effect of reserpine, tetrabenazine and syrosingopine on emesis in cats induced by peruvoside and ouabain.

Drug	*Dose (µg/kg i.v.)*	*Control*	*No. of cats vomited/No. of cats tested* *Reserpine* *i.m.*	 *i.c.v.*	 *Tetrabenazine* *i.v.*	 *Syrosingopine* *i.v.*
Peruvoside	125	20/20	0/6	0/3	0/6	0/6
Ouabain	60	15/15	0/6	0/3	0/6	0/6
Copper sulphate 20 mg in 50 ml of water	orally	8/8	12/12	6/6	12/12	12/12

i.v. = intravenous; i.c.v. = intracerebroventricular.
Doses of reserpine, tetrabenazine and syrosingopine given in Table 1.

Table 4 Effect of mepyramine maleate, BOL–148, phenoxybenzamine and chlorpromazine on emesis in cats induced by peruvoside and ouabain.

Drug	*Dose µg/kg*	*Control*	*No. of cats vomited/No. of cats tested* *Mepyramine maleate* *i.m.* *10 mg/kg*	 *i.c.v.* *3 mg*	 *BOL–148* *i.v.* *50 µg/kg*	 *i.c.v.* *20 µg*	 *Phenoxybenzamine* *i.v.* *5 mg/kg*	 *i.c.v.* *2 mg*	 *Chlorpromazine* *i.v.* *2.5 mg/kg*	 *i.c.v.* *750 µg*
Peruvoside	125	20/20	6/6	3/3	6/6	3/3	5/10	5/5	6/6	3/3
Ouabain	60	15/15	6/6	3/3	6/6	3/3	5/5	6/6	6/6	3/3
Copper sulphate 20 mg in 50 ml of water	orally	8/8	–	–	–	–	5/5	–	–	–

i.v. = intravenous; i.c.v. = intracerebroventricular; i.m. = intramuscular.

with an average latency of 3 min (range 2-4 minutes).

Pimozide in a dose of 1 mg/kg intravenously, 24 h prior to emetic challenge protected the cats against peruvoside-induced emesis. All cats rendered refractory to the emetic action, vomited after oral administration of copper sulphate.

Effect of prior treatment with SKF-525-A The effects of haloperidol (1 mg/kg i.v.) given 6 h and 24 h after administration of SKF-525-A (75 mg/kg i.p.) are presented in Table 6. The most effective antiemetic dose of haloperidol (1 mg/kg i.v.) failed to protect about 30% of the animals at 6 hours. The antiemetic action of haloperidol was not seen in SKF-525-A treated animals when haloperidol was administered 24 h after SKF-525-A treatment; all animals in the group which received ouabain and 6 out of 7 cats in the group challenged with peruvoside vomited although they had received haloperidol just prior to emetic challenge.

Effect of phenobarbitone treatment The effect of chronic administration of sodium phenobarbitone on the antiemetic action of haloperidol is shown in Table 7.

Haloperidol in a dose of 250 µg/kg intravenously did not have any antiemetic action in normal animals. However, after pretreatment with phenobarbitone this dose of haloperidol afforded protection in 50% of the animals when challenged with the emetic dose of either peruvoside or ouabain.

Discussion

The role of catecholamines in the vomiting mechanism is not clearly defined. High concen-

Table 5 Antiemetic action of haloperidol in cats.

			No. of cats vomited/No. of cats tested		
Drug	*Dose (µg/kg i.v.)*	*Control*	*Haloperidol-treated*		*Pimozide-treated*
			i.v. (1 mg/kg)	*i.c.v. (200 µg)*	*i.v. (1 mg/kg)*
Peruvoside	125	20/20	0/10	3/3	0/3
Ouabain	60	15/15	0/10	3/3	—
Copper sulphate 20 mg in 50 ml of water	orally	8/8	20/20	—	3/3

i.v. = intravenous; i.c.v. = intracerebroventricular.

Table 6 Effect of SKF-525-A treatment on the protective action of intravenous haloperidol against vomiting in cats induced by peruvoside and ouabain.

Drug	*Dose (µg/kg)*	*Route*	*No. of cats vomited/No. of cats tested** *6 h*	*24 h*
Peruvoside	125	i.v.	1/3	6/7
Ouabain	60	i.v.	1/3	7/7

* = All animals received SKF-525-A, (75 mg/kg i.p.) followed by haloperidol (1 mg/kg i.v.) (see text).

Table 7 Effect of sodium phenobarbitone treatment on the antiemetic action of intravenous haloperidol in cats.

			No. of cats vomited/No. of cats tested	
			Haloperidol (250 µg/kg i.v.)	
Drug	*Dose (µg/kg)*	*Route*	*Before treatment*	*After treatment**
Peruvoside	125	i.v.	5/5	5/10
Ouabain	60	i.v.	5/5	4/8

* = Animals treated with sodium phenobarbitone (25 mg/kg i.p.) for 8 days prior to challenge.

tration of amines occurs in the area postrema (Vogt, 1954). Adrenaline and its precursors potentiate apomorphine-induced emesis (Boyd & Cassel, 1957; Forster & Gunther, 1962). Further, catecholamine depleting drugs have been reported to afford protection against emesis induced by 5-hydroxytryptophan (Cahen, 1964), apomorphine (Boyd & Cassel, 1957), and staphylococcal enterotoxin (Sugiyamma, Bergdoll & Wilkerson, 1960).

Our results indicate that reserpine, tetrabenazine and syrosingopine abolish vomiting induced by peruvoside and ouabain. Since reserpine depletes catecholamines from both central and peripheral adrenergic neurones (Carlsson, Rosengren & Nilsson, 1957), the role of catecholamines was further analysed by the use of intravenous tetrabenazine or syrosingopine and intraventricular reserpine. Tetrabenazine has been shown to exert most of its actions centrally (Quinn, Shore & Brodie, 1959). The results obtained by treatment with tetrabenazine intravenously or intracerebroventricular reserpine indicate that the depletion of catecholamines from the brain is responsible for antagonizing the emesis induced by peruvoside and ouabain. The protective action of these drugs does not seem to be due to sedation of the animal, since all cats that failed to vomit after peruvoside or ouabain readily vomited after oral copper sulphate indicating the emetic mechanism or threshold to copper sulphate was unimpaired.

Our results with syrosingopine are intriguing. Syrosingopine has been shown to deplete catecholamines peripherally (Orlans, Finger & Brodie, 1960). It antagonized both peruvoside and ouabain-induced vomiting. In our experiments ouabain has been shown to produce vomiting by activating the chemoreceptor trigger zone (Borison & Brizzee, 1951), while peruvoside induces vomiting by an action on the nodose ganglion (Gaitonde & Joglekar, 1972). The antiemetic action of syrosingopine in peruvoside-induced emesis could well be due to depletion of amines in the nodose ganglion which has been reported to be rich in adrenergic neurones (Koelle, 1955). The effectiveness of syrosingopine in ouabain-induced emesis may be due to depletion of catecholamines in the chemoreceptor trigger zone.

Phenoxybenzamine afforded only partial protection against peruvoside-induced emesis; five out of ten animals treated with the blocking agent did not vomit. Further, the drug was totally ineffective against ouabain-induced emesis. Lack of protection after phenoxybenzamine in this case may indicate that the drug did not reach the receptor site. Alternatively more than one transmitter mechanism may be involved in the integrated process of vomiting. BOL-148 and antihistamines were ineffective in peruvoside- and ouabain-induced vomiting, suggesting that 5-hydroxytryptamine and histamine do not play a role in the mechanism of vomiting. Chlorpromazine, although it produced a fair degree of sedation, failed to abolish the emetic action of either ouabain or peruvoside.

Haloperidol (1 mg/kg i.v.) abolished peruvoside- and ouabain-induced emesis, in spite of the fact that the animals received the maximal effective emetic dose. Although haloperidol produced sedation, impairment of emesis did not seem to be due to this action. The vomiting mechanism in these animals is not impaired, as can be seen from the fact that all the haloperidol-treated animals vomited after oral copper sulphate. Haloperidol is thus an effective antiemetic agent in peruvoside- and ouabain-induced emesis.

One fifth of the effective intravenous antiemetic dose of haloperidol when given intracerebroventricularly, failed to protect the animals from emesis induced by peruvoside and ouabain. This may indicate that intracerebroventricular haloperidol may not reach the receptor site. However, another possibility is that a metabolite of the drug may be responsible for the antiemetic activity of haloperidol. Such a possibility is suggested by our experiments, in which microsomal enzyme inhibitors or inducers were used. Thus in cats pretreated with SKF-525-A, a known microsomal enzyme inhibitor (Brodie, 1956) the protective action of haloperidol was completely lost, when the drug was administered 24 h after SKF-525-A treatment, with some effect possibly being present, even at 6 h after treatment with SKF-525-A. Further evidence for implication of a metabolite of haloperidol is provided by our experiments in which phenobarbitone, a microsomal enzyme inducer (Brodie, 1956) was used. After chronic treatment of cats with sodium phenobarbitone, haloperidol in a dose as small as 250 μg/kg intravenously, was found to produce an antiemetic action in 50% of the cats treated. This dose of haloperidol failed to protect normal animals (Table 7). Thus both the microsomal enzyme inhibitor as well as the inducer influenced the antiemetic action of haloperidol in a predictable manner. We therefore postulate that a metabolite of haloperidol in cats is probably an effective antiemetic agent.

Haloperidol is a weak adrenoceptor blocking drug but a strong dopamine antagonist (Fuxe & Sjoqvist, 1972; Van Rossum, 1966). Apomorphine has also been shown to stimulate dopamine receptors. Haloperidol seems to protect effectively against apomorphine- and digitalis-induced emesis and it is therefore possible that digitalis- as well as apomorphine-induced emesis, may be due to activation of dopamine receptors. Pimozide has been reported to be a specific dopamine antagonist, (Fuxe & Sjoqvist, 1972). Cats treated with pimozide (1 mg/kg) 24 h prior to emetic challenge, were totally protected. This lends further evidence to the possibility of involvement of dopamine receptors in the nodose ganglion and chemoreceptor trigger zone responsible for emesis induced by peruvoside or ouabain. Since one of the earliest manifestations of digitalis toxicity is vomiting, haloperidol and pimozide may be useful clinically in the treatment of vomiting due to digitalis toxicity.

The authors wish to thank The Director, Central Council for Research in Indian Medicine and Homoeopathy, New Delhi, India, for financial assistance and Dr P. Janssen, Janssen Pharmaceutica, Belgium for the generous supply of haloperidol and pimozide.

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Br. J. Pharmac. (1975), 54, 163-170

HYPERGLYCAEMIA PRODUCED BY DRUGS WITH ANALGESIC PROPERTIES INTRODUCED INTO THE CEREBRAL VENTRICLES OF CATS

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1 The effects on blood glucose of four substances with analgesic properties (apomorphine, pethidine, codeine and etorphine) and of prostaglandin E_1 were examined in unanaesthetized cats. They were applied by the intraventricular route being either injected into a lateral ventricle or infused into the fourth ventricle through implanted Collison cannulae.

2 Apomorphine gave rise to pronounced hyperglycaemia in a dose of 0.75 mg which produced scarcely any hyperglycaemia on intravenous injection. It was more effective on infusion into the fourth ventricle than on injection into a lateral ventricle and was approximately half as potent as morphine in provoking hyperglycaemia.

3 Codeine produced no hyperglycaemia in doses of 0.75 and 1.5 mg.

4 Pethidine had a weak hyperglycaemic action in doses of 0.75 and 1.5 mg, but the effect was not regularly obtained. Potency of the drug was at most only a third to a sixth that of morphine.

5 Etorphine produced strong hyperglycaemia on infusion into the fourth ventricle in a dose of 10 µg. Unlike apomorphine or morphine it was more potent on injection into a lateral ventricle when it produced a strong hyperglycaemic response in doses of 5 or 1 µg, which were subthreshold on infusion into the fourth ventricle. However, this response may have been brought about indirectly as a result of severe asphyxia and of convulsions associated with the injections. On infusion into the fourth ventricle, etorphine was about 75 times as potent as morphine in producing hyperglycaemia.

6 Prostaglandin E_1 had no hyperglycaemic action when infused into the fourth ventricle in a dose of 400 ng.

Introduction

Morphine injected into the cerebral ventricles of unanaesthetized cats produces hyperglycaemia, an effect first described by Borison, Fishburn, Bhide & McCarthy (1962). The hyperglycaemia is a central effect, but it does not stem from an action on structures in the walls of the third ventricle as suggested by these authors. Recent experiments indicate that the structures on which morphine acts when producing this effect are situated near the ventral surface of the brain stem caudal to the trapezoid bodies and are reached after the drug has passed into the subarachnoid space through the lateral recesses (Feldberg & Shaligram, 1972; Feldberg & Gupta, 1974; Dey, Feldberg & Wendlandt, 1975).

In the present experiments, four other substances with analgesic properties were examined to find out whether they produce hyperglycaemia on injection into a lateral ventricle or on infusion into the fourth ventricle of unanaesthetized cats, and whether there is some correlation between analgesic and hyperglycaemic action. The four substances were apomorphine, pethidine, codeine and etorphine. The first three are less potent analgesics than morphine, whereas etorphine is more potent.

An effect on blood glucose has previously been found with three of them, pethidine, apomorphine and codeine, but only by subcutaneous injection in rabbits. Pethidine in doses up to 20 mg/kg was without effect (Schaumann, 1940) but hyperglycaemia was observed with apomorphine and codeine. Campbell & Morgan (1973) obtained hyperglycaemia with 5 mg apomorphine and attributed the effect to a central action because it was abolished by anaesthesia. Ko (1935) found that apomorphine-induced hyperglycaemia was dose-dependent in doses ranging from 0.5 to 20 mg/kg, that the effect was reduced after bilateral splanchnicotomy and almost abolished by removal of the suprarenals. Hyperglycaemia followed the injection of 2 to 20 mg/kg codeine and its potency was less than that of morphine (Akimoto, 1933). The effect was abolished after section of the splanchnic nerves or removal of the suprarenals (Ro, 1935/36) as well as by x-ray radiation of the animals (Sai, 1941).

Finally, experiments were carried out with prostaglandin E_1 because Bergström, Carlson & Orö (1966) had found that in dogs, intravenous infusions of this substance at rates of 0.2, 0.4, 0.8 and 1.6 μg kg^{-1} min^{-1} effected a slight increase in blood glucose concentration although the change was statistically significant only with 0.8 μg kg^{-1} $minute^{-1}$. Further, the suggestion has been made that inhibition of effects of prostaglandin may be involved in morphine actions; this possibility has been discussed in connection with the analgesic action of morphine and its derivatives because compounds belonging to this group were found to inhibit the stimulating action of prostaglandins E_1 and E_2 on cyclic adenosine 3′,5′-monophosphate (AMP) formation in rat brain (Collier & Roy, 1974). On the other hand, both morphine and apomorphine stimulate prostaglandin synthetase derived from bull seminal vesicles or rabbit brain (Collier, McDonald-Gibson & Saeed, 1974).

Methods

Cats of either sex weighing between 3 and 3.8 kg were used. For injections into the left lateral ventricle or for infusions into the fourth ventricle, a Collison cannula was implanted in an aseptic operation under pentobarbitone sodium anaesthesia. The method for cannulating the lateral ventricle was that described by Feldberg & Shaligram (1972), and for the fourth ventricle, that of Feldberg & Gupta (1974). The injections or infusions of drugs were made after recovery from operation, a few days later, without anaesthesia. They were injected into the cannulated lateral ventricle in a volume of 0.15 ml, followed immediately by an injection of 0.05 ml of a 0.9% w/v NaCl solution (saline), or infused in a volume of 40 μl by a microinfusion pump which delivered this volume from a 100 μl syringe in 4 min 20 seconds. Details of the infusion procedures are given by Dey *et al.* (1975). Intravenous injections were made through a saphenous vein.

The actual position of cannulae implanted into lateral or fourth ventricles and the delivery of drugs through them was ascertained at the end of the experiments by injecting or infusing a 0.8% bromophenol blue solution of the same volume as that used for injecting or infusing the drugs through the cannulae. The cats were killed 20 min later during pentobarbitone sodium anaesthesia and the position of the cannulae and the spread of the dye observed with the naked eye. On infusion into the fourth ventricle, no dye appeared to have entered the aqueduct as there was no staining of the peri-aqueductal grey matter; however, there was deep staining of the anterior half of the floor of the fourth ventricle which contrasted with the unstained posterior half. The stained area ceased abruptly in a straight line between the two lateral recesses. There was staining of the ventral surface of the brain stem and cerebellum.

The collection of blood samples from the inferior vena cava and the estimation of their glucose content by a glucose oxidase method were as described previously (Feldberg & Shaligram, 1972).

Drugs

The following drugs were used: morphine sulphate, pethidine hydrochloride (Roche Products Ltd., Welwyn Garden City), codeine phosphate, apomorphine hydrochloride (MacFarlane Smith Ltd., Edinburgh), etorphine hydrochloride (a gift from Dr J.W. Lewis, Reckitt & Colman, Hull). All drugs were dissolved in saline immediately before use. This was particularly important for apomorphine as the solution turned a greenish colour within 10 min and was then inactive. The amounts of the drugs given in the text refer to the salts. Prostaglandin E_1 was a gift from Dr J. Pike, Upjohn & Co., Kalamazoo, Michigan, U.S.A.

Results

Apomorphine

A dose of 0.75 mg produced a strong hyperglycaemic response when injected into a lateral ventricle or infused into the fourth ventricle, but had scarcely any effect on blood glucose level when injected intravenously. The effect is thus a central one. The results obtained in five cats are summarized in Table 1. They show that apomorphine was more effective when infused into the fourth ventricle than when injected into a lateral ventricle. In the three experiments on cats 1 and 2 following injections into a lateral ventricle, the blood glucose concentration rose to between 140 and 146 (mean 143) mg/100 ml, whereas in the three experiments on cats 3, 4 and 5, where infusions were made into the fourth ventricle, it rose to between 183 and 247 (mean 219) mg/100 ml. The last two experiments of the Table show further that 0.75 mg of apomorphine had scarcely any hyperglycaemic action after intravenous injection. This difference in efficacy of

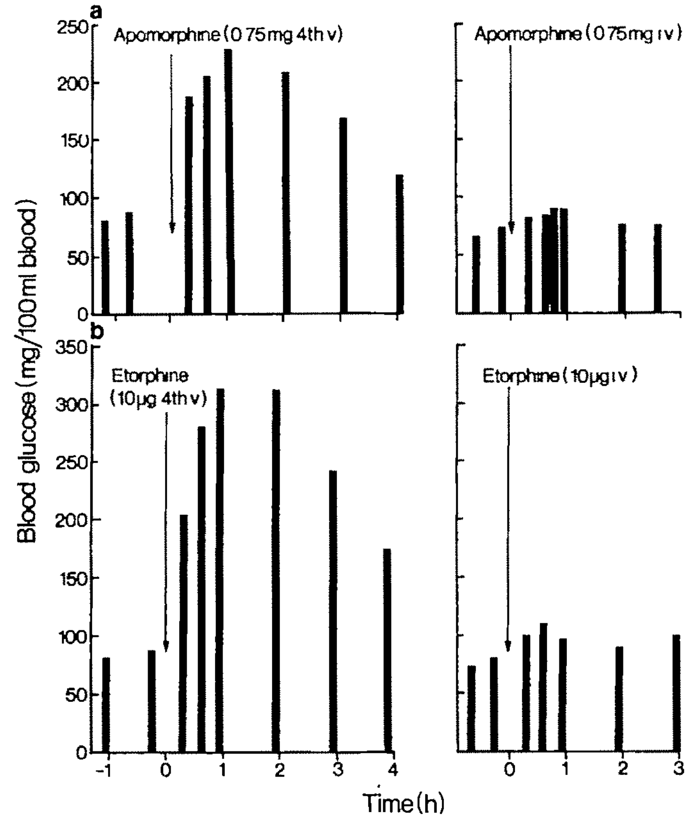

Figure 1 Effects on blood glucose in two unanaesthetized cats. (a) Effect of apomorphine (0.75 mg) infused into fourth ventricle (4th v) and three days later injected intravenously into the same cat. This is cat no. 4 of Table 1. (b) Effect of 10 µg etorphine infused into fourth ventricle (4th v) and 24 h later injected intravenously (i.v.). This is cat no. 14 of Table 2. In this and in Figure 2, the arrows (↓) indicate moment of infusion or injection and the vertical columns time, as shown on the abscissae, before and after introduction of the drugs when venous blood samples were taken. Glucose concentration is given by the height of the columns.

Table 1 Effect of apomorphine (0.75 mg) on blood glucose concentration in unanaesthetized cats

		Blood glucose (mg/100 ml). Min before and after apomorphine administration							
Cat no.	*Route of administration*	*−90 to −45*	*−46 to −5*	*+20*	*+40*	*+60*	*+120*	*+180*	*+240*
1	Lateral ventricle	100	102	143		136	136	136	
1	Lateral ventricle	73	73	140	146	144	92	92	
2	Lateral ventricle	80	88	132	140	132	108	92	
3	IVth ventricle	89	95	174	183	117	130	109	
4	IVth ventricle	80	88	188	206	228	208	168	120
5	IVth ventricle	95	87	213	227	247	182	140	
2	Intravenous	78	70	74	74	76	82	85	
4	Intravenous	64	72	80	84	88	76	76	

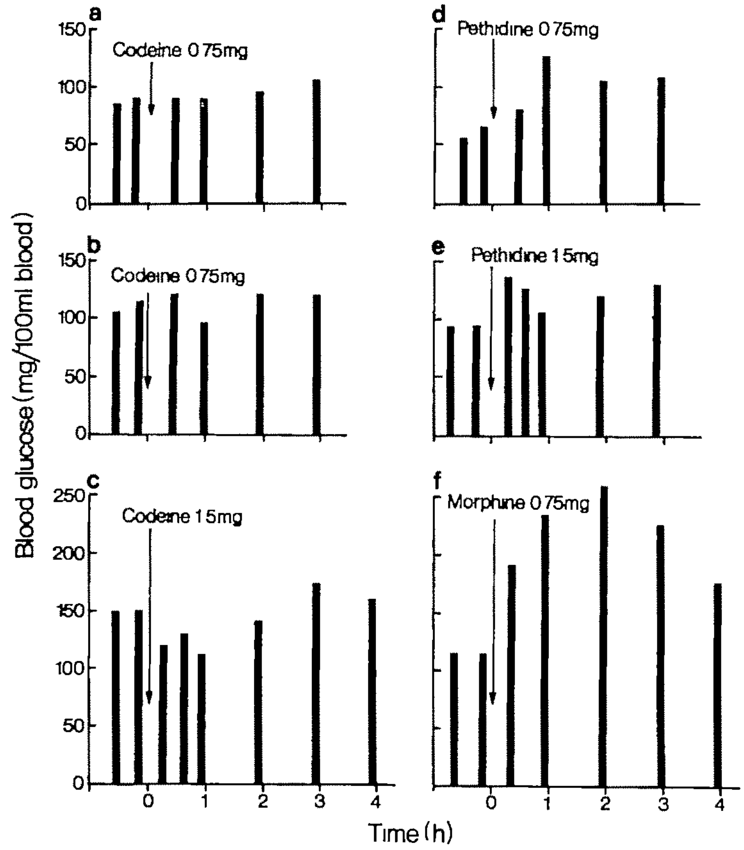

Figure 2 Effects on blood glucose in five unanaesthetized cats (a) and (d) from the same cat. (a), (b) and (c), Effect of 0.75 and 1.5 mg of codeine, (d) and (e) of pethidine, and (f) of 0.75 mg morphine.

apomorphine introduced into the cerebral ventricles or injected intravenously is also illustrated in Figure 1a. In this cat (no. 4 of Table 1) the drug was first infused into the fourth ventricle and three days later injected intravenously.

When the results are compared with those previously obtained with morphine injected into a lateral ventricle (Feldberg & Shaligram, 1972), it would appear that apomorphine was approximately half as potent in producing hyperglycaemia because 0.75 mg apomorphine was less potent than 0.75 mg, but more potent than 0.24 mg of morphine.

It is known that cats, compared to dogs, are relatively insensitive to the emetic action of apomorphine (Reynolds & Randall, 1957). In the present experiments, 0.75 mg produced the full emetic response consisting of salivation, retching and vomiting in one, and salivation with retching in another cat after injection into the lateral ventricle. Salivation alone occurred also on infusion of apomorphine into the fourth ventricle, began within a few minutes of the injection or infusion and continued for 20 to 45 minutes. During this period the cats were restless and two cats defaecated and urinated. Shivering was regularly produced but began late, 20 to 25 min after the injection or infusion when the periods of restlessness and salivation were over. It then continued for another 20 to 45 minutes.

Codeine and pethidine

Figure 2 illustrates five experiments in which these compounds were injected into a lateral ventricle in a dose of 0.75 or 1.5 mg. Experiments (a), (b) and (c), show that these doses of codeine did not produce hyperglycaemia; in experiments (b) and (c), the injections actually resulted in a transient mild hypoglycaemic state. Pethidine had a weak hyperglycaemic action which was not regularly obtained. In experiment (d), a mild effect was obtained with 0.75 mg but doubling the dose, as in experiment (e), did not increase it. In two other experiments, the infusion of 1.5 mg into the fourth ventricle produced mild hyperglycaemia in one animal, and none in the other. The mild hyperglycaemia resulting from 0.75 and 1.5 mg in experiments (d) and (e) was of about the same order as that previously observed after 0.24 mg morphine similarly injected (Feldberg & Shaligram, 1972). The hyperglycaemia-producing property would appear therefore to be not more than about a third to a sixth that of morphine.

Experiment (f) of Figure 2 shows, for comparison, the strong hyperglycaemic effect obtained on injection of 0.75 mg morphine hydrochloride into a lateral ventricle. As the sulphate salt of morphine was used in all previous experiments to produce hyperglycaemia on intraventricular injection (Borison *et al.*, 1962; Feldberg & Shaligram, 1972; Feldberg & Gupta, 1974; Dey *et al.*, 1975), this experiment, performed with the hydrochloride, shows that hyperglycaemia is not an effect of the sulphate moiety but of morphine itself.

Of the various behavioural effects seen after intraventricular injection of morphine, only two, shivering and miaowing, occurred with codeine and pethidine, and for much shorter periods than with morphine. With codeine, shivering began about 1 min and with pethidine about 5 min after injection. At first, shivering was strong, widespread and continuous, but within 10 to 25 min it became weaker, intermittent, and then stopped. Miaowing occurred only with the higher dose (1.5 mg). It began about 5 min after codeine and about 20 min after pethidine injection, and lasted for 10-12 minutes. At first, the response was strong and occurred frequently but it became gradually weaker, and the intervals between each miaowing increased before it finally stopped.

Etorphine

Hyperglycaemia was obtained with etorphine in much smaller doses than with apomorphine or morphine, and the drug was more potent on injection into the lateral ventricle than on infusion into the fourth ventricle. On injection into the former, 5 μg and even 1 μg were sufficient to produce a strong hyperglycaemic response, but on infusion into the latter, these doses were subthreshold and it required 10 μg to demonstrate the hyperglycaemic effect. The high potency of etorphine as well as its relative difference in potency when administered by the two routes are evident from the first thirteen experiments shown in Table 2; seven were performed with injections into the lateral ventricle, and six with infusions into the fourth ventricle. The last two experiments of the Table show further that an intravenous injection of 10 μg etorphine, a dose which produced a strong hyperglycaemic response when introduced into either ventricle, had merely a mild hyperglycaemic effect. Figure 1b illustrates this difference in efficacy on the same cat after 10 μg etorphine had first been infused into the fourth ventricle, and 24 h later injected intravenously.

The hyperglycaemic effect of 10 μg etorphine infused into the fourth ventricle was of the same order as previously found for 0.75 mg morphine (Feldberg & Gupta, 1974) given by the same route. Under these conditions, etorphine thus

Table 2 Effect of etorphine on blood glucose concentration in unanaesthetized cats

			Min before and after etorphine administration							
Cat no.	*Route of administration*	*Dose (μg)*	*−90 to −45*	*−46 to −5*	*+20*	*+40*	*+60*	*+120*	*+180*	*+240*
6	Lateral ventricle	10	69	76	162	190	157	162	119	
7	Lateral ventricle	10	82	89	227	262	266	217	111	
8	Lateral ventricle	10	111	115	271	315	320	302	235	
9	Lateral ventricle	10	83	79	262	285	333	228	124	
10	Lateral ventricle	5	113	102	258	267	283	220	108	
11	Lateral ventricle	5	100	92	275	350	308	275	171	
11	Lateral ventricle	1	—	88	176	170	170	—	—	
12	IVth ventricle	10	89	89	156	180	208	172	98	
13	IVth ventricle	10	76	82	192	224	228	228	140	88
14	IVth ventricle	10	80	87	204	278	313	313	243	174
15	IVth ventricle	5	63	60	71	95	92	112	83	
12	IVth ventricle	5	88	96	100	102	100	104	88	
13	IVth ventricle	2	84	82	82	86	90	88	100	
14	Intravenously	10	73	82	100	111	95	89	100	
16	Intravenously	10	83	92	100	119	117	120	125	

appears to be about 75 times more potent than morphine.

There were also differences in behavioural response depending on site of introduction. Certain effects were produced when 1, 5 or 10 μg etorphine were injected into a lateral ventricle, but did not occur when these doses were infused into the fourth ventricle. They included severe slowing of respiration leading to asphyxia, short bouts of tonic-clonic convulsions and extreme rigidity of spasm of skeletal muscles lasting for several minutes, greatly impeding respiratory movements and thereby aggravating the asphyxia. The hyperglycaemia produced on injection of etorphine into a lateral ventricle in doses which were subthreshold on infusion into the fourth ventricle, may well have resulted directly from these effects.

Other effects obtained with 10 μg and sometimes with 5 μg etorphine on introduction into either ventricle were constriction of the ear vessels, mild shivering and ataxia lasting about 10 min, and mydriasis and restlessness of longer duration. During the initial period of ataxia, circling movements occurred with the hindquarters flexed so that the cat moved on its belly. Later the animal moved about continuously looking from side to side, and repeatedly jumping against the roof or side of the cage. When jumping against the side of the cage, it clung to it and remained immobile for a few seconds before it fell down. All movements were rapid. Mydriasis quickly became maximal, remained so for about an hour and subsided gradually during the following two hours. The cats did not react to squeezing the paws, suggesting strong analgesia. Some of these effects were also observed after intravenous injection of 10 μg of etorphine which, in addition, produced intense salivation.

Respiratory depression was also obtained when doses larger than 10 μg were infused into the fourth ventricle. When 20 μg were administered in this way, respiration stopped and artificial ventilation by rhythmic compression of the thorax with both hands was applied for 10 to 20 min.

Prostaglandin E_1

Infusions of 400 ng of prostaglandin E_1 into the fourth ventricle produced no hyperglycaemia but merely strong shivering, the usual effect of this drug when given by the intraventricular route.

Discussion

The present experiments show that apomorphine, pethidine and etorphine, but not codeine, share with morphine the property of producing hyperglycaemia when applied by the intraventricular route; the hyperglycaemia is a central effect because it was obtained with doses which were ineffective on intravenous injection. This was shown for apomorphine, but was not examined for pethidine as it has only a weak and irregular hyperglycaemic effect. No experiments were done to determine the site of action of these substances although it is probably the same as that on which morphine acts when producing its hyperglycaemic effect, i.e. the ventral surface of the brain stem (Dey *et al.*, 1975). When injected or infused into the cerebral ventricles it therefore has to pass through the lateral recesses into the subarachnoid space before it can act. The finding that morphine is more effective when infused into the fourth ventricle instead of being injected into a lateral one is also readily explained by such a site of action. In the present experiments, apomorphine was also more effective on infusion into the fourth, than on injection into a lateral ventricle. However, the reverse was true for etorphine. On injection into a lateral ventricle, it produced hyperglycaemia in doses that were subthreshold on infusion into the fourth ventricle. However, this hyperglycaemia is probably brought about indirectly through the severe asphyxia and/or convulsions produced by these injections. Otherwise two sites of action would have to be proposed, one on the ventral surface being the site where apomorphine and morphine act, and another in the walls of the cerebral ventricles rostral to the fourth ventricle, a site not acted upon by either drug.

When comparing the hyperglycaemic potency of the four analgesic drugs examined with that previously obtained for morphine when injected into a lateral or infused into the fourth ventricle (Feldberg & Shaligram, 1972; Feldberg & Gupta, 1974), it would appear that apomorphine is about half, and pethidine at most about a third to a sixth as potent as morphine. With codeine no hyperglycaemia was obtained but if its potency were about a tenth that of morphine, no effect would have been detected with the sensitivity of the method used. On the other hand, etorphine was much more potent than morphine and, based on the results obtained after infusion into the fourth ventricle, it would appear to be about 75 times more potent.

Thus it is evident that there is some correlation between hyperglycaemia and analgesic action of these substances, for apomorphine, pethidine and codeine are less potent analgesics, and etorphine is much more potent than morphine.

To correlate the two actions is justified because analgesia, too, appears to be produced by an action on structures on the ventral surface of the brain stem. However, to assess the relative potencies of the two actions of the various substances more quantitatively, it would be necessary to compare them in the same species and with the same method of administration.

The evidence for an action on the ventral surface of the brain stem is similar for the analgesic as for the hyperglycaemic action although the structures involved are unlikely to be the same. Herz, Albus, Metys, Schubert & Teschemacher (1970) studied in rabbits the analgesic effect of morphine injected into different parts of the ventricular system. They found it to be most effective when injected into the aqueduct or fourth ventricle and concluded that morphine produced analgesia by an action on structures in their walls. They did not envisage the possibility of an action on structures at or near the ventral surface of the brain stem, reached after the drug had passed into the subarachnoid space through the lateral recesses. Recently the high potency of morphine in producing analgesia when introduced into the fourth ventricle was demonstrated in cats, but it was further noted that morphine was as effective when infused into the subarachnoid space beneath the ventral surface of the brain stem, although it did not enter any part of the ventricular system (Dey & Feldberg, 1975). It was therefore concluded that the analgesic effect of morphine when given by the intraventricular route, and in fact when given intravenously, also resulted from an action on structures situated near the ventral surface of the brain stem. Although this may be a main site of action, it is probably not the only one.

The importance of species differences is seen from the results obtained by Blane, Boura, Fitzgerald & Lister (1967) when they compared the analgesic action of subcutaneous injections of etorphine with that of morphine in different species. Etorphine was about 850 times more potent than morphine in mice, about 1700 times more potent in rats and about 8600 times more potent in guinea-pigs. In man the analgesic action of etorphine appears to be about 1000 times greater than that of morphine (Blane & Robbie, 1970) although no exact comparison has been made. A table compiled by Jaffe (1965) from various publications compares the relative analgesic potencies of pethidine and codeine on subcutaneous injection in man to that of morphine. The analgesic action of pethidine is given as an eighth to a tenth, and of codeine as a twelfth that of morphine. Apomorphine is not used clinically as an analgesic and data are not available for a quantitative comparison with morphine.

The importance of route of administration is seen from results obtained by von Cube, Teschenmacher, Herz & Hess (1970). They compared the antinociceptive action of etorphine and morphine in rabbits; when injected intravenously, etorphine was nearly 10,000 times as potent, but injected intraventricularly only about 70 times as potent as morphine. This ratio of about 70 to 1 is practically the same as that observed during the present experiments on cats for the hyperglycaemic action of etorphine and morphine infused into the fourth ventricle: the ratio was about 75 to 1. Thus it may well be that a comparison employing both the same route of administration and the same species would reveal a close correlation between the two activities of the analgesic drugs examined in the present paper.

P.K.D. is a Wellcome Research Fellow.

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(Received December 24, 1974.
Revised February 6, 1975)

Br. J. Pharmac. (1975), 54, 171-179

ALTERNATIVE APPROACHES TO ANALGESIA: BACLOFEN AS A MODEL COMPOUND

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1 It is suggested that analgesia could be produced by drug action at the spinal level through (a) interference with neurotransmission at primary afferent terminals; (b) enhancement of the 'gate control' of the sensory input to the spinal cord mediated through descending spinal tracts; or (c) increased presynaptic inhibition of primary afferents by a direct action.

2 Baclofen (9.4-70.3 μmol/kg, i.p.), which may mimic spinal presynaptic inhibition, produced a dose-dependent increase in the response times of mice in a hot-plate test, but high doses also impaired motor function.

3 Morphine hydrochloride (5.3-40 μmol/kg, i.p.) increased the response time of mice in the hot-plate test and had little effect on motor function.

4 Combination of baclofen (9.4 or 23.4 μmol/kg) with morphine (13.3 μmol/kg) produced greater increases in response time than either drug administered alone but with little concurrent effect on motor function.

5 The possibility that baclofen may have some analgesic action and a potentiating effect on other analgesics is discussed.

Introduction

The major classes of analgesic agents in current clinical use have a number of drawbacks. The narcotic analgesics, although potent, have several undesirable actions and continued use may lead to dependence. This latter hazard is not encountered with the antipyretic analgesics but these agents are generally more limited in terms of analgesic potency. Structural modification of the narcotic analgesics has produced some advances but an alternative approach to the design of analgesics would be an attractive proposition and may prove to be more fruitful. One such approach would be to modify the sensory input to the central nervous system at the spinal level thereby reducing the transmission of information related to a painful stimulus to higher centres. This approach would clearly present a problem of selectivity of drug action but three possible mechanisms of action might be considered:

(1) Interference with neurotransmission at primary afferent terminals

The logical development of drugs having a selective action at sites in the spinal cord almost certainly depends on the identification of the neurotransmitters involved in sensory pathways and the associated synaptic events. The substance which is currently most favoured as a candidate for a neurotransmitter released by primary afferent terminals is glutamic acid (Hammerschlag & Weinreich, 1972; Johnson, 1972) although the evidence is not yet convincing and this amino acid has not been specifically linked with one or more groups of primary afferent fibres. An additional candidate is the polypeptide Substance P which is found in high concentrations in dorsal roots and has more powerful excitatory effects on spinal neurones than glutamic acid (Otsuka, Konishi & Takahashi, 1972a,b; Konishi & Otsuka, 1974).

If either of these compounds in time proves to be an excitatory neurotransmitter released by primary afferent neurones which convey pain-related information, then the discovery of drugs which reduce the postsynaptic availability or postsynaptic interaction of the transmitter might constitute an important advance in the development of alternative analgesics. The finding of Haldeman & McLennan (1972) that glutamic acid diethylester blocks the excitatory action of iontophoretically applied glutamic acid and also synaptic activation of dorsal horn interneurones in the cat spinal cord and in the thalamus (Haldeman, Huffman, Marshall & McLennan, 1972) is an interesting development in this respect.

(2) Enhancement of the 'gate control' of the afferent input to the spinal cord mediated through descending spinal tracts

Melzack & Wall (1965) have suggested that the afferent impulses which trigger pain reactions pass through a gate control mechanism whose effectiveness is determined by a balance of the activity in large and small cutaneous afferent fibres and by impulses descending from higher centres. The gating control is believed to be operated through presynaptic inhibition of primary afferent terminals and has been shown to be particularly effective in reducing the activity of receptive interneurones of (Rexed, 1952) laminae IV and V (Wall, 1967; Hillman & Wall, 1969). There is accumulating evidence to indicate that morphine analgesia is in part associated with an increase in the activity of tryptaminergic pathways descending from the dorsal raphe to inhibit the discharge of lamina IV and V cells in the spinal cord (Vogt, 1974). Stimulation of the dorsal raphe produces profound analgesia in anaesthetized or conscious cats (Liebeskind, Guilbaud, Besson & Oliveras, 1973; Oliveras, Besson, Guilbaud & Liebeskind, 1974) and a similar response in rats was reduced by the administration of *p*-choloro-phenylalanine (Akel, Mayers & Liebeskind, 1972).

Increasing by pharmacological means the post-synaptic availability of the presumed neurotransmitter, 5-hydroxytryptamine, in the descending control of the spinal gating mechanism could therefore be an alternative approach to analgesia.

(3) Increase of presynaptic inhibition of afferent neurones by a direct action

There is considerable experimental support for the suggestion that γ-aminobutyric acid (GABA) is a neurotransmitter released by neurones which mediate presynaptic control of primary afferent terminals. Spinal GABA concentrations are highest in dorsal horn (Graham, Shank, Werman & Aprison, 1967; Johnston, 1968) and levels are reduced by chronic dorsal root section which suggests a close association of GABA with primary afferent terminals (Gottesfeld, Kelly & Rayner, 1973; Jones, Jordan, Morton, Stagg & Webster, 1974). Furthermore, presynaptic inhibition (inferred from dorsal root potentials) initiated from inputs to various levels of the cat central nervous system (CNS) is antagonized by bicuculline (Benoist, Besson, Conseiller & Le Bars, 1972; Benoist, Besson & Boissier, 1974) which also blocks the effects of GABA on spinal neurones (Curtis, Duggan, Felix & Johnston, 1971). Levy & Anderson (1974) have additionally suggested that primary afferent hyperpolarization may be GABA-mediated since it may be blocked by bicuculline and picrotoxin. GABA has been shown to depolarize primary afferent terminals thereby mimicking presynaptic inhibition (Eccles, Schmidt & Willis, 1962; 1963; Barker & Nicoll, 1972; Levy, 1974; Nishi, Minota & Karczmar, 1974) although these studies have concentrated on the presynaptic control of monosynaptic reflexes. However, if GABA has a similar role in the control of cutaneous afferents, then an analgesic effect might be achieved by increasing the availability of GABA (or related compound) at receptors on afferent terminals.

GABA does not itself cross the blood-brain barrier but the *p*-chlorophenyl substituted derivative (baclofen) readily does so and is used clinically for the treatment of spasticity (Lioresal, CIBA). There is evidence to suggest that it may reduce sensory input to the cord by mimicking presynaptic inhibition (Pierau & Zimmerman, 1973) although this view has been questioned by Curtis, Game, Johnston & McCulloch (1974).

The possibility remains that if baclofen were found to mimic presynaptic inhibition of the afferent input of pain-related information, then this compound might have analgesic properties. The results of clinical trials on patients suffering from spastic conditions certainly indicate that the drug afforded considerable relief from the pain which accompanied the condition (Pinto, Polikar & Debono, 1972). However, it is difficult to determine whether this effect is solely the result of reduced muscle contracture.

Since baclofen is apparently relatively specific to the spinal cord in its action, (Koella, 1972) it was an obvious candidate for initial investigations towards possible alternative analgesic agents. We have therefore studied the effect of baclofen on the response of mice to a noxious (thermal) stimulus and compared the effects of this drug with those of morphine. Since animal tests can show only whether the response of the subject to a noxious stimulus is altered by drug treatment, we prefer to use the term antinocisponsive action rather than analgesic or antinociceptive action to describe a drug-induced delay of the response.

Methods

Experiments were performed on male Swiss Albino mice weighing between 25 and 30 g. They were moved to the laboratory at least one hour before starting the experiment.

Antinocisponsive test

Each test was performed as follows. The mouse was lifted by the tail and placed on a hot plate (55°C ± 0.5°C) and covered by a 250 ml glass beaker. A stop watch was started as the hind paws made contact with the hot-plate and stopped as soon as an end-point was observed, at which time the animal was removed and the response time recorded in hundredths of 1 minute. Two behavioural end-points were accepted. The first was shaking of one or both hind paws and the second was bringing the hind paw into contact with the mouth. If an end-point had not been reached after 0.60 min (the 'cut-off point'), the animal was removed from the hot-plate to prevent damage to the paws. The use of a beaker to cover the animal ensured that the hind paws were in continuous contact with the hot-plate since the animal's normal reaction to this environment was to rear up against the wall of the beaker. This method was found to give consistent end-points in pilot experiments. A control reading was taken for each animal before drug treatment and then at 15 or 30 min intervals after drug treatment.

Tests of motor function

Motor function was tested in each animal by placing it on a horizontal wooden bar (2.5 cm diameter) which rotated at 20 rev minute^{-1}. An untreated mouse when placed on the upper aspect of the bar was able to remain in this position by means of forward locomotion for a period of 1 minute. A mouse with impaired motor co-ordination was unable to maintain this position and in an extreme case, it would fall off the bar. Intermediate cases, however, would turn passively with the bar. The number of times that an animal rotated with the bar was used as the basis for the first test of motor function. The duration, d, of the test (normally 1 min) was recorded together with r the number of times the animal rotated passively with the bar. Since the bar rotation rate was 20 rev min^{-1}, the maximum number of passive rotations that a mouse could undergo was 20. The score assigned to each animal was given by $20 - r$. Occasionally, an animal would fall off the bar in less than one minute. In such a case, a second test was conducted and frequently a value for d of 1 min was achieved. On the few occasions where the mouse failed to remain on the bar for 1 min in the second test, r was computed from $r \times 1/d$ and this value was used to compute the score for that animal. Changes in performance following drug treatment are expressed as Δp (= mean post injection score − mean pre-injection score). A positive value indicates an improved performance and a negative value a deterioration.

A second test was applied immediately after the first. If a normal mouse was reorientated on the bar such that it would have to walk backwards in order to maintain its position on the upper aspect of the bar, it would simply return to its former orientation. The ability of treated and untreated mice to perform this manoeuvre was scored on a simple pass or fail scheme.

These tests were performed on each animal before drug treatment and at 30 min intervals thereafter. In addition to the standardized tests each animal was closely observed for changes in behaviour.

Drug treatment

Drug treatments were assigned on a random basis within each group of animals. Each animal was weighed immediately before the start of the experiment and all drug concentrations were such that the injection volume was 10 ml/kg. Saline (0.9% w/v NaCl solution) controls were administered on a similar basis. The experiments were performed 'blind' in that the experimenter was not aware of the drug treatment given to individual animals.

Drugs were dissolved in 154 mM sodium chloride and administered intraperitoneally. Doses of morphine hydrochloride and baclofen (Lioresal, CIBA) are stated as μmol/kg.

Results

Antinocisponsive effects of morphine and baclofen

Four dose levels of each drug were tested and both morphine and baclofen were found to increase the response time for mice in the hot-plate test. Examples of results for two treatment groups are illustrated in graphical form in Figure 1. The time dependence of response time in each treatment group was tested by Friedman two way analysis of variance (Null hypothesis − the reaction times of the mice receiving a given treatment were independent of the time at which they were tested). Probabilities derived from this test are shown in Table 1. In five of the drug treatments the responses varied significantly ($P < 0.05$) with time but for the lowest dose of each drug, the null hypothesis could be rejected with less certainty and similarly for 26.6 μmol/kg morphine. Analysis of the results for saline-treated animals showed that the reaction times were independent of the time at which they were measured and the

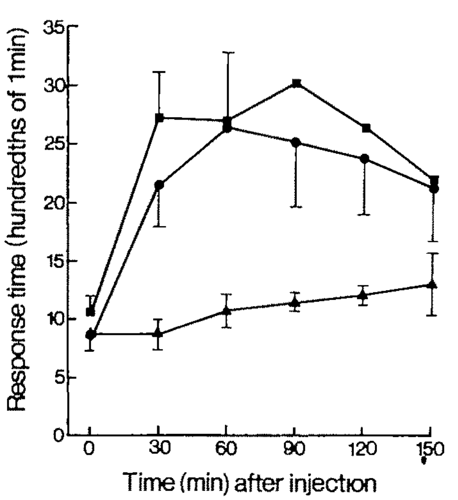

Figure 1 Reaction times of mice in hot-plate test determined at 30 min intervals. The first point on each curve is the pre-injection control reading which served as the lower limit for the determination of 'total effects' (see text). (■) Morphine, 26.6 μmol/kg; (●) baclofen, 23.4 μmol/kg; (▲) saline, 10 ml/kg. Each point is the mean result from 6 animals. Standard errors are not included where one or more members of the group reached the cut-off point.

apparent upward trend of the response (Figure 1) was not significant.

The antinocisponsive effects of the two drugs have been computed by two methods:

(a) The total antinocisponsive effect for the pooled results from each group was determined as follows. The response time (in hundredths of 1 min) was plotted against time (in min) after administration of the drug as shown in Figure 1.

Table 1 Probability that the reaction times in the hot-plate test measured throughout the course of the experiment are independent of the time at which they were measured.

Drug	*Dose (µmol/kg)*	P
Morphine	5.3	< 0.20
	13.3	< 0.05
	26.6	< 0.10
	40.0	< 0.01
Baclofen	9.4	< 0.20
	23.4	< 0.05
	46.9	< 0.05
	70.3	< 0.02
Saline	10.0 (ml/kg)	< 0.50

Results for each treatment group of 6 animals were analyzed by the Friedman Two-Way Analysis of Variance.

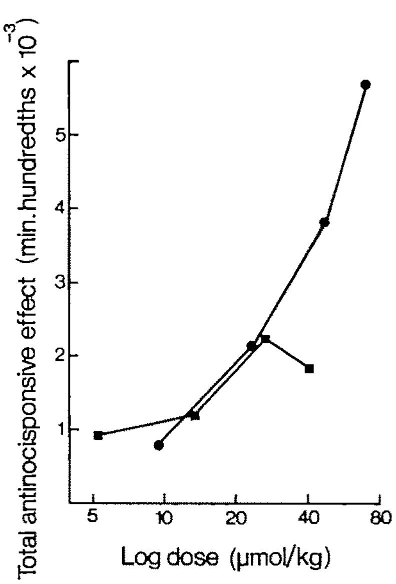

Figure 2 Log dose-response curves for the total antinocisponsive effects of baclofen (●) and morphine (■). Each point is the mean result from 6 animals. Results, when analyzed by the Kruskall-Wallis one-way analysis of variance (Null hypothesis — there is no difference between total effects of the four dose levels of morphine) showed no significant ($P < 0.05$) difference ($P < 0.1$). However, if the lowest dose is omitted from the analysis, $P < 0.05$. Equivalent value for the four doses of baclofen is $P < 0.001$).

The area delineated by the graph and a horizontal line drawn from the pre-injection control response was determined and this value (expressed in min hundredths) was used as an index of the total antinocisponsive effect. This method is analogous to that of Winter & Flataker (1950).

Log dose-response curves constructed on the basis of calculated total effects are shown in Figure 2. Both drugs produced dose-dependent increases in the reaction time although the highest dose of morphine (40 μmol/kg) appeared to be less effective than 26.6 μmol/kg. The antinocisponsive effect of baclofen consistently increased with dose and the two higher doses produced greater total effects than any dose of morphine.

(b) Peak effects — since the time course of action of the two drugs varied and the onset of effects in individual animals was somewhat variable, the measured peak effects of the treatments occurred at different times. Peak effects for individual animals have therefore been determined and the mean for each treatment group plotted as log dose-response curves for the two drugs, (Figure 3). The curve for morphine again indicates

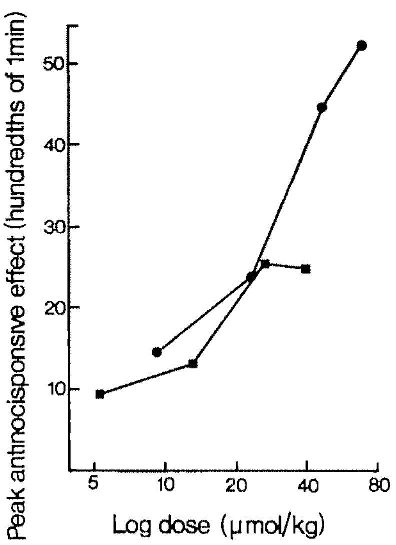

Figure 3 Log dose-response curves for the peak antinocisponsive effects of morphine (■) and baclofen (●). Kruskall-Wallis analysis of the full dose range of each drug yields $P < 0.02$ and $P < 0.001$ for morphine and baclofen respectively. Each point is the mean from 6 animals.

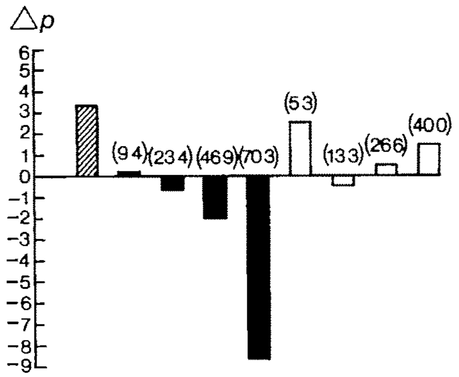

Figure 4 Performance of mice in rotation test of motor function. A positive index of performance Δp, indicates improvement relative to the pre-injection control score. A negative value of Δp indicates a deterioration. Figures in brackets are doses in μmol/kg. Each column is the mean score of a treatment group of 6 animals. Hatched columns — saline (10 ml/kg); solid columns — baclofen; open columns — morphine.

that the response reaches a maximum at the 26.6 μmol/kg dose level. In contrast, baclofen produced a dose-dependent increase in peak effect, throughout the dose range.

Motor function tests

Motor function as denoted by the performance of each treatment group in the rotation test is shown in Figure 4. The highest dose of baclofen (70.3 μmol/kg) produced a severe depression of performance in this test. Lower doses had a less marked effect but only the lowest dose (9.4 μmol/kg) gave a positive index of performance (Δp). Positive values of Δp were obtained for all morphine treatments except for the 13.3 μmol/kg dose which slightly impaired performance. All drug treatments resulted in Δp values less than that obtained for saline controls.

In the reorientation test, only two drug treatments produced a marked change in performance relative to saline controls, most scores remaining near the 100% level. Baclofen 70.3 μmol/kg impaired performance such that re-orientation was successfully accomplished in only 35% of 36 attempts, whereas 46.9 μmol/kg baclofen reduced the score to 76%.

It was found repeatedly that the two highest doses of baclofen were associated with exophthalmos and piloerection which suggests that the drug may have a direct or indirect action on the autonomic nervous system. In addition, these two treatments often reduced spontaneous activity and in some animals receiving the highest dose there was a degree of catalepsy. Some of these animals could be rolled on to their dorsal surface and after a few seconds in this position, they would suddenly right themselves.

Effects of mixtures of baclofen and morphine

Since low doses of baclofen apparently had an antinocisponsive action without obvious impairment of motor function, it seemed worthwhile to determine whether a more powerful antinocisponsive effect could be achieved by the administration of a low dose (9.4 or 23.4 μmol/kg) of baclofen together with a low dose of morphine (13.3 μmol/kg). The effect of administration of these mixtures was compared with that of the individual doses administered alone and the usual saline controls. Log dose-response curves based on total antinocisponsive effects are shown in Figure 5. The effects of the mixture were found to be greater than those observed with individual doses of either drug in this experimental series. Furthermore, the 23.4 μmol/kg baclofen plus 13.3 μmol/kg morphine mixture produced a greater effect than any single dose of morphine in the first experimental series. The antinocisponsive effect of the two drugs administered as a mixture

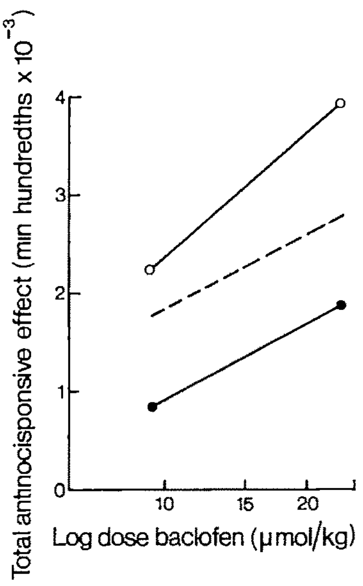

Figure 5 Log dose-response curves for baclofen alone (●) and in combination with morphine 13.3 μmol/kg (o). The dotted line indicates the theoretical curve derived from addition of the total effects of baclofen and morphine administered as separate treatments. Each point is the mean total antinocisponsive effect of groups of six animals tested at 15 min intervals over a period of 150 minutes. Statistical analysis, (Kruskall-Wallis) showed that combination of morphine with baclofen produced an increase in the effect relative to baclofen alone. The higher dose of mixture or of baclofen alone produced a greater effect than the corresponding lower doses ($P < 0.01$ in all cases).

thus appears to be at least additive. In fact, if the dose-response relationship for the mixtures is compared with that predicted from simple addition of the total effects for baclofen and morphine administered alone (dotted line in Figure 5) there is a suggestion of potentiation.

Log dose-response curves derived from peak effects in individual mice confirm the additive effects of the two drugs when administered as a mixture but 'potentiation' is apparent only at the lower dose level. This discrepancy is due to the fact that four of the animals that received the higher dose of mixture reached the cut-off point in the hot-plate test without giving a definite end-point. The cut-off point is more limiting in peak effect than in total effect determinations since the latter are based on responses monitored over a period of 150 min whereas peak effects are calculated from single observations, the maximum value of which is pre-set.

A striking feature of the effects of the mixtures is that they produce marked antinocisponsive actions but neither dose caused a deterioration in performance in the rotation test. In fact, both mixture treatments were associated with small positive Δp values.

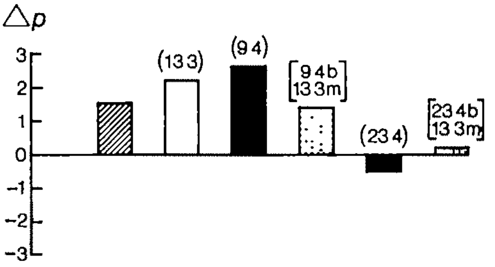

Figure 6 Rotation test performance for mice treated with mixtures or morphine or baclofen alone. Indices of performance expressed as for Figure 4. Hatched columns — saline (10 ml/kg); open columns — morphine; solid columns — baclofen; dotted columns — baclofen (b) plus morphine (m).

A similar pattern was reflected in the re-orientation test where both doses of mixture were without effect relative to saline.

All mice in this second experimental series seemed normally active and coordinated from subjective assessment. Thus, in contrast to some of the mice that received high doses of baclofen in the first experimental series, there was no obvious disability to which increased reaction times in the hot-plate tests could be attributed. However, it should be noted that during testing on the hot-plate, some animals treated with a mixture were apparently distressed by the environment in that they made attempts to escape from the confines of the beaker although they had not given a recognised end-point in the test. In control observations of these animals with the plate removed from the heat source this behaviour was not seen. This suggests that the observed distress response was associated in some way with the perception of heat (or possibly humidity above the water bath) although, as already indicated, there was nothing to suggest that the animals were unable to respond normally in the hot-plate test.

Discussion

The aim of this study was to determine whether baclofen has an antinocisponsive effect which might be related to an analgesic action. The determination of analgesic effects in animals is a difficult procedure since it is necessary to discriminate between an inability to respond to a noxious stimulus and a genuine analgesia. We have therefore attempted to determine the effects of morphine and baclofen on motor function in mice by means of two standardized tests together with subjective assessment of behaviour (albeit in 'blind' trials). The large increases in reaction times in the hot-plate test observed with 46.9 and 70.3 μmol/kg baclofen were associated with a deterioration in performance in the motor function tests. The apparent 'analgesic' effect of these two treatments was therefore probably due to a reduced ability to execute an end-point response. Tests with animals treated with lower doses of baclofen (9.4 and 23.4 μmol/kg) did not reveal any marked deterioration in performance although the possibility remains that the motor function tests were not sufficiently sensitive to detect subtle changes. However, none of the animals treated with these low doses of baclofen showed any of the cataleptic or other behavioural effects seen at higher dose levels. Also, animals receiving low doses generally gave clear end-point responses in the hot-plate test which gave the impression that it may be possible to obtain some selectivity of action towards antagonism of perception of or response to a noxious stimulus without markedly affecting motor function.

The important difference between the effects of the drug combinations and those of baclofen administered alone is that the combinations afforded a powerful antinocisponsive action without a decrease in performance in the motor function tests, although our reservation that some animals showed some distress during the hot-plate test should be reiterated.

This study was based on the premise that an increase in presynaptic inhibitory control over sensory inputs to the spinal cord would result in an analgesic effect. What evidence is there to suggest that the marked antinocisponsive action is mediated through presynaptic inhibition? The possibility that GABA is a presynaptic inhibitory neurotransmitter implies that an increased availability of GABA at receptors on primary afferent neurones would damp down the sensory input. Pierau & Zimmerman (1973) have provided indirect evidence from intracellular recording of motoneurones that intravenously administered baclofen mimics presynaptic inhibition in the spinal cord. This suggestion has recently been challenged by Curtis *et al.*, (1974) since they were unable to demonstrate antagonism by biculline of the inhibitory effects of iontophoretically applied baclofen on spinal neurones. Bicuculline has previously been shown to antagonize presynaptic inhibition (Levy, Repkin & Anderson, 1971; Benoist *et al.*, 1972) and the effects of GABA in the cat spinal cord (Curtis *et al.*, 1971). Curtis *et al.* (1974) found that the uptake of 10^{-8}M L-glutamic acid into brain slices was reduced by 34% in the presence of baclofen (10^{-4}M) and that 'GABA transaminase activity' was reduced by 35% in the presence of 10^{-3}M baclofen. On the basis of these observations, they suggest that of the GABA receptors present in the CNS, baclofen possibly only interacts with those of GABA transaminase and the reduction *in vitro* of high affinity L-glutamic acid uptake does not account for the depression of neuronal firing by baclofen. However, estimations based on the data of Faigle & Keberle (1972) suggest that the CNS concentrations of baclofen achieved in rats after intravenous administration of a high dose (47 μmol/kg) are of the order of 5×10^{-6}M. It seems unlikely therefore that the pharmacological action of baclofen is primarily the result of inhibition of GABA transaminase or of glutamic acid uptake.

We find that the dosage of baclofen is extremely critical. Effects may vary from slight to severe depression of reflexes with concurrent respiratory depression or even, in some cases, a paradoxical phase of hyperirritability, all occurring within a relatively small dose range. It is difficult therefore to relate the CNS concentrations associated with given behavioural effects with those encountered locally during iontophoretic application of baclofen. If presynaptic inhibitory connections are the site of action of intravenously administered baclofen as suggested by Pierau & Zimmerman (1973), it is important to determine whether primary afferent depolarization is altered by this compound and whether the synaptic activation of dorsal horn interneurones is modified in a fashion identical with that normally associated with increased presynaptic inhibition of pain related afferent input to the spinal cord.

Selectivity of action is a fundamental requirement for an analgesic having a spinal locus of action. Although baclofen is unlikely to prove to be an ideal analgesic, its relative specificity to the spinal cord (Koella, 1972) and its potent antinocisponsive action, particularly in combination with morphine, render it worthy of further study. Even if it is not an analgesic in doses which do not produce depression of spinal reflexes it might be of use as an adjunct to other analgesics or in certain aspects of spinal anaesthesia.

We feel that analgesic action at a spinal level is a useful approach and we would welcome the development of compounds which may be utilized in this way.

We are indebted to Mr F.W. Ballhatchet for his construction of the apparatus, to Dr A.M. Jukes (Medical Adviser, C.I.B.A. Laboratories) for the provision of baclofen, and to Dr R.A. Webster for his critical appraisal of the manuscript.

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(Received September 30, 1974)

Br. J. Pharmac. (1975), 54, 181-188

CHARACTERIZATION OF AN INHIBITORY RECEPTOR IN RAT HIPPOCAMPUS: A MICROIONTOPHORETIC STUDY USING CONFORMATIONALLY RESTRICTED AMINO ACID ANALOGUES

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1 Pyramidal cells in rat hippocampus were used to study the molecular dimensions of a receptor for inhibitory amino acids in the central nervous system. The inhibitory potencies of γ-aminobutyrate (GABA), β-alanine and glycine were compared by standard microiontophoretic techniques. Subsequently, rigid cyclopentane and cyclohexane amino acid analogues were applied by iontophoresis and their relative efficacies were compared with those of the naturally occurring amino acids.

2 GABA was the most effective of the small aliphatic amino acids in producing inhibition of the firing of hippocampal pyramidal neurones. β-Alanine was less effective and glycine was the least effective. GABA-induced inhibition was antagonized by concurrent iontophoresis of picrotoxin or bicuculline, whereas strychnine did not antagonize GABA inhibition.

3 The ability of the series of substituted aminocyclopentane and aminocyclohexane carboxylic acids to produce inhibition of pyramidal cells was a direct function of the separation of amino and carboxylic acid groups. In both series of the cyclic amino acids the most potent inhibition was demonstrated when the spatial separation was similar to that of the extended GABA molecule (4.74 Å). Additionally, the inhibition of hippocampal pyramidal cells by (±)-*cis*-3-amino-cyclopentanecarboxylic acid, like that produced by GABA, could be blocked by simultaneous application of picrotoxin or bicuculline, but not by strychnine.

4 The present results suggest that the physiologically active conformation of GABA is the fully extended molecule, and additionally indicate that one dimension of the postsynaptic receptor site is within the range of 4.2 to 4.8 ångströms.

Introduction

Previous neurophysiological studies have demonstrated the existence of postsynaptic inhibitory mechanisms in the pyramidal cells of the mammalian hippocampus (Andersen, Eccles & Loyning, 1964). γ-Aminobutyric acid (GABA) has been demonstrated to mimic this postsynaptic inhibition in the hippocampus (Curtis, Felix & McLennan, 1970) and in other test systems within the mammalian central nervous system (CNS) (Obata, Ito, Ochi & Sato, 1967; Galindo, 1969; Curtis, Duggan, Felix & Johnston, 1971; Curtis, Duggan, Felix, Johnston & McLennan, 1971; Bruggencate & Engberg, 1971). β-Alanine and glycine have also been suggested as possible inhibitory neurotransmitters in various CNS systems (Bruggencate & Engberg, 1971) but a detailed comparative study of their effect on the pyramidal cells of the hippocampus specifically has not been reported.

GABA in aqueous solution can exist in numerous molecular conformations, and the spatial separation of amino and carboxy groups can range from 2.4 to 4.8 Å (McGeer, McGeer & McLennan, 1961; Steward, Player, Quilliam, Brown & Pringle, 1971; Van Gelder, 1971; Gilardi, 1973). A previous study using analogues of GABA to characterize the conformation of GABA which is physiologically active on the inhibitory stretch receptor of the crayfish suggests that the optimal spatial separation of the amino and carboxy groups is 4.0 Å or more (McGeer *et al.*, 1961), a separation most consistent with an extended conformation of GABA. Recent studies by Beart, Curtis & Johnston (1971) using a conformationally-restricted analogue, 4-aminotetrolic acid, also suggest that the extended form of GABA is the physiologically active conformation. However, studies of the GABA molecule in solid state crystals indicate a spatial separation within the range of 2.7 to 3.1 Å (Steward *et al.*, 1971). Van Gelder (1971), on the basis of theoretical calculations, has suggested that the active conformation is a folded form of the molecule.

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The present study represents an attempt to characterize the physiologically active conformation of GABA and thereby indirectly delineate the dimensions of its synaptic receptor site. The present experimental approach utilizes a series of aminocyclohexane carboxylic (ACHC) and aminocyclopentane carboxylic (ACPC) acids. Preliminary findings with the series of cyclohexane amino acids have been reported (Segal, Sims, Maggiora & Smissman, 1973). These results served to illustrate the feasibility of using this experimental approach. However, the incomplete rigidity of the ACHC amino acid did not allow precise definition of the receptor site. For example, several conformers of the same ACHC compound may exist which have markedly different spatial separations of amino and carboxy functions (Table 1). The advantage of the series of ACPC compounds is the rigid and planar cyclopentane ring which holds a constant distance between amino and carboxy functional groups. The ACPC which most exactly mimics the potency of GABA can then be presumed to have the same spatial separation of amino and carboxy groups as in the physiological conformation of GABA; thus, one dimension of the GABA receptor site can be determined. An additional important characteristic of the series of amino acids analogues on which we are currently reporting is that there are no functional groups (e.g., unsaturated bonds or aromatic rings) which would alter the chemical nature of the amino or carboxy moieties.

Methods

Adult (150-250 g) male Zivic Miller rats were used. The rats were initially anaesthetized with 3.5% halothane, tracheotomized and then placed in a stereotaxic apparatus. Anaesthesia was maintained with 0.5-1.0% halothane. A 3 mm diameter hole was trephined in the skull above the dorsal hippocampus (3 mm caudal to the bregma and 2 mm lateral to the midline). The meninges were removed and 3% agar (40° C) in Ringer solution was applied to the exposed cortex. In some experiments the cortex was first removed by suction, then the alveus was exposed and the space was filled with the agar solution. Rectal temperature was monitored continuously and maintained between 36-38° C. Cerebral electrical activity was recorded using the central barrel (3M NaCl, 2-4MΩ, overall tip diameter = 4-6 μm) of a five barrel micropipette, amplified and displayed on an oscilloscope. Extracellular unitary potentials were discriminated from background noise and smaller spikes using a voltage gating device and the voltage output was then integrated at one-second intervals. These one-second values were then plotted as rate of cell firing.

Three of the outer four barrels of the five barrel micropipette were filled with three of the following compounds: 0.5 M GABA (Sigma); 0.5 M β-alanine (Sigma); 0.5 M glycine (Fisher); 10 mM strychnine hydrochloride (Aldrich); 10 mM picrotoxin (Mann), 10 mM bicuculline (Pierce); 1.0 M acetylcholine hydrochloride (Sigma); or one of the aminocyclohexane carboxylic acid or aminocyclopentane carboxylic acid compounds (synthesized by Dr L. Maggiora, University of Kansas, Department of Medicinal Chemistry). The ACPC and the ACHC compounds were dissolved in 0.9% w/v NaCl solution; the racemic compounds were prepared to a concentration of 1.0 M and the non-racemic compounds to 0.5 M. The pH of the resultant solutions ranged between 6.4-7.0 and the compounds were injected as cations.

The fourth outer barrel was filled with 3 M NaCl and used for ejecting neutralizing current (Salmoiraghi & Weight, 1968). A constant current solid-state circuit provided the currents needed to eject or retain the compounds in the barrels, and the circuit automatically neutralized tip currents (Geller & Woodward, 1972).

One of the difficulties inherent in an iontophoretic study is the variability in transport number among different pipettes (Hoffer, Neff & Siggins, 1971). To minimize any effect of pipette variability as a cause of the observed differences among the effects of the various compounds, each compound was tested in at least four micropipettes (Segal & Bloom, 1974). Additionally, the results obtained for each group of compounds were compared statistically using analysis of variance tests.

In testing the relative potency of the individual amino acids, a 10 s pulse (unless otherwise specified) was applied at 1 min intervals. The amount of current needed to produce complete inhibition was first determined and then current was sequentially decreased from a suprathreshold level at each successive pulse until no effect was observed. Two variables were measured, the threshold for a complete blockade of unitary firing and the amount of current needed to reduce spontaneous firing to one-half of the maximal rate. Several of the compounds tested did not produce

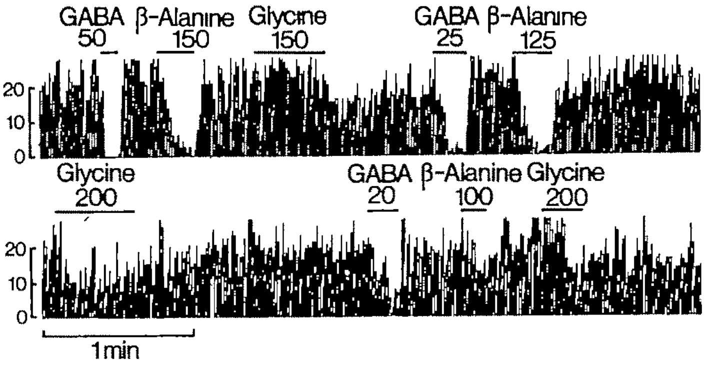

Figure 1 Effects of iontophoretic release of aliphatic amino acids on spontaneous activity of a hippocampal cell. Unit activity was integrated over 1 s intervals. The bar underneath each compound represents the duration of the ejection interval. The numerical value represents the current in nanoamperes (nA) needed to produce the observed change in firing rate. All compounds in this and the following figures were ejected as cations. The ordinate value in this and subsequent figures represents spikes per s intervals.

complete blockade; therefore, current for half-maximal inhibition was chosen as the major variable for comparisons among the compounds.

Results

Cell distribution

A total of 214 cells (15 rats) in the dorsal hippocampus were studied. The majority of the cells was located in a narrow zone of stratum pyramidalea (0.2-0.3 mm ventral to the alveus, 1.9-2.1 mm deep to the surface of the neocortex) of CA1, CA2 and CA3 regions (Lorente de Nó, 1934). Some of the cells were located in area CA3 of the lower blade of dorsal hippocampus (1.2-1.4 mm below the alveus). No differences were observed among the cells in the different regions with respect to sensitivity to the aliphatic and cyclic amino acids tested. Cellular activity was

Table 1 Characteristics of the investigated compounds and summary of the experimental data

	Distance in Å between amino and carbonyl group	*Number of cells*	*Mean current*	σ_X
A. *Natural amino acids*				
Glycine	2.35	20	163.9	20.6
β-Alanine	3.66	21	80.0	7.0
γ-Aminobutyric acid	4 74	65	19.7	1.0
B. *Cyclopentanecarboxylic acids*				
1-amino	2.39	12	221.0	24.2
(±)-*cis*-2-amino	2.54	16	179.3	16.21
(±)-*trans*-2-amino	3.50	10	75.0	6.3
(±)-*cis*-3-amino	4.08	20	18.5	3.1
(±)-*trans*-3-amino	4.77	14	8.9	2.4
C. *Cyclohexane carboxylic acids*				
1-amino	2.39	12	220.8	24.2
(±)-*cis*-2-amino	2.81	30	205.2	15.9
(±)-*trans*-2-amino	2.70*, 3.47**	31	146.3	15.1
(±)-*cis*-3-amino	4 81*, 2.46**	26	94.0	10 6
(±)-*trans*-3-amino	4.04	16	256.2	20.4
(±)-*cis*-4-amino	4.62	10	282.5	17.5
(±)-*trans*-4-amino	5.58*, 4.35**	10	***	***

* equatorial equatorial, ** axial axial; *** largely ineffective

not recorded from other layers in the hippocampus, (c.f. Herz & Nacimiento, 1965). The cells studied were spontaneously active within a range of 1-20 spikes per second; however, in some cases leakage of acetylcholine was used to increase the firing rate (Krnjević, Mitchell & Szerb, 1963).

Responses to small aliphatic amino acids

The response to GABA was tested in 65 cells. The mean current necessary to produce half-maximal inhibition of firing after 10 s was 19.7 nA (s.e. mean = ± 1.0). The responses exhibited a uniform, characteristic short latency (about 1 s), and frequently, a rebound excitation would follow termination of the iontophoretic current (Figure 1 and Table 1). The response to β-alanine was tested on 21 cells and complete suppression of firing was difficult to demonstrate. The mean current needed to evoke half-maximal inhibitory responses was 80.0 nA, (s.e. mean = ± 7.0). The other small aliphatic amino acid, glycine, was tested on 20 cells. Only a very slight inhibition was observed following glycine iontophoresis and both a large current (163.9 nA, s.e. mean ± 20.6) and a long ejection pulse (up to 1 min) was needed to produce half-maximal inhibition. The difference in mean current required for half-maximal responses among the three amino acids was statistically significant by analysis of variance, ($F_{2,103}$ = 34.86; $P < 0.001$).

Responses to cyclopentane amino acids

The responses to iontophoretic application of (±)-1,3-*trans*-ACPC was determined in 14 cells. This compound was the most potent cyclopentane amino acid inhibitor of spontaneous firing of hippocampal cells among all the compounds tested (Figure 2). The mean current for half-maximum response was 8.9 nA (s.e. mean = ± 2.4) and the inhibition persisted for 5 to 15 s after termination of the ejecting current (Figure 3). The effect of (±)-1,3-*cis*-ACPC was tested on 20 cells. The mean current for half-maximal response was 18.5 ± 3.1 nA and this compound resembled its 1,3-*trans*-counterpart in that inhibition persisted after termination of ejection current (Figure 3), although for a shorter time (2-5 seconds).

The other ACPC compounds were much less effective in inhibiting the firing of hippocampal pyramidal cells (Figure 3). (±)-1,2-*trans*-ACPC (tested on 10 cells) produced half-maximal inhibition at a higher mean ejection current of 75.0 nA (s.e. mean = ± 6.3).

The (±)-1,2-*cis*-ACPC, tested on 16 cells, was even less pofent (mean current for half maximal inhibition = 179.3 nA, s.e. mean = ± 16.21). The 1,1-ACPC was the least effective cyclopentane amino acid and its effect was observed only after large currents (12 cells; mean current for half maximal inhibition = 221 nA, s.e. mean = ± 24.2) and only after the current was sustained for long intervals (Figure 3). The difference among the effects of at least some of the cyclopentane amino acids was highly significant ($F_{4,67}$ = 59.57; $P < 0.001$).

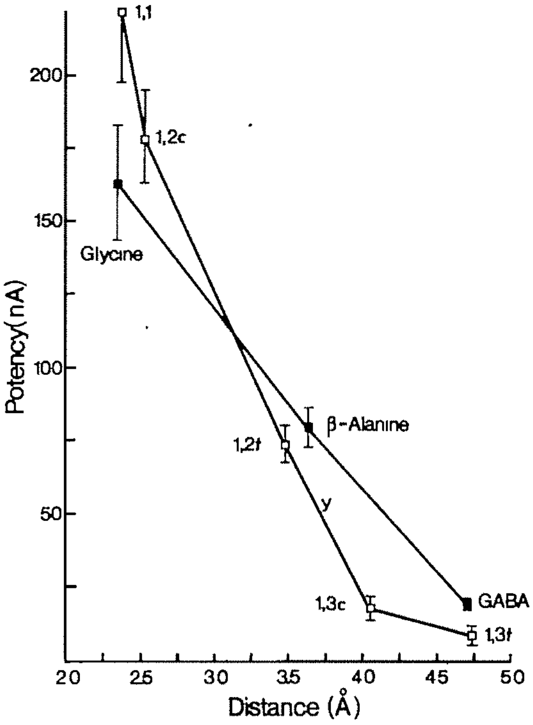

Figure 2 Comparison of the potency of the aminocyclopentane carboxylic (ACPC) amino acids with those of the small aliphatic amino acids tested. The ordinate values represent current required for half-maximal inhibition. The abscissa value is the distance in ångstroms between the amino and carboxy functions (value for the aliphatic compounds represent the value for the fully extended molecule). The results indicate a direct correlation between potency and geometric separation of amino and carboxy groups.

Responses to cyclohexane amino acids

The ACHC compounds were far less active than the ACPC series (Figure 4). The (±)-1,3-*cis*-ACHC derivative was the most effective of the cyclohexane amino acids (Table 1). It produced, in 26 cells, half-maximal inhibitions when ejected with a mean current of 94.0 nA (s.e. mean = ± 10.6).

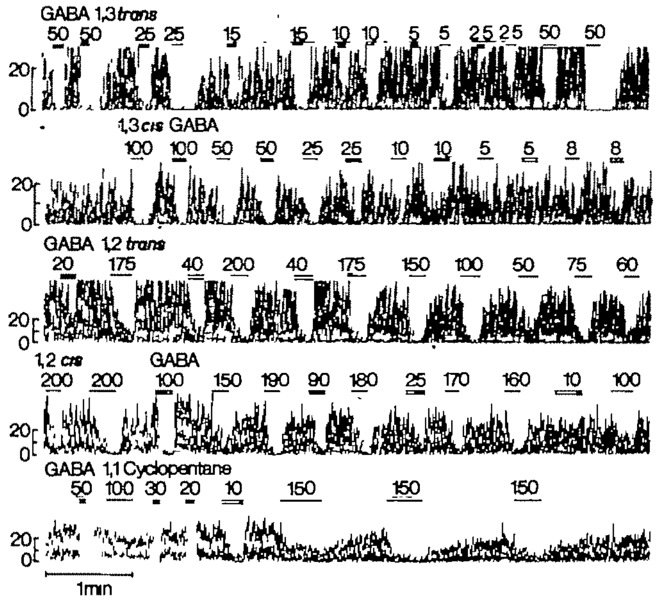

Figure 3 Comparison between the effects of γ-aminobutyric acid (GABA) and the aminocyclopentane carboxylic compounds on firing rates of hippocampal cells. Unitary activity was maintained in these experiments by concurrent continuous application of acetylcholine. No interaction between acetylcholine and the compounds tested on the activity of the cells tested was observed. Note the prolonged inhibitory action of the (±)-1,3-*trans* and 1,3-*cis* cyclic amino acids on the activity of the cells. GABA application is indicated by the double bars delineating the duration of application.

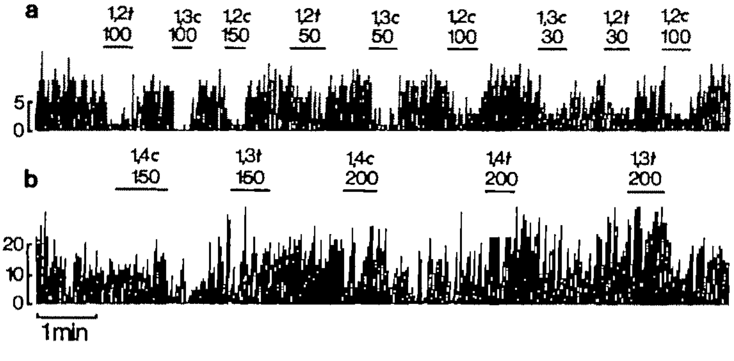

Figure 4 Responses to the aminocyclohexane carboxylic amino acid series. The two traces were taken from two different experiments. Both traces were taken from spontaneously active cells (i.e., no acetylcholine was used to enhance unitary activity).

Next in efficacy was the (±)-1,2-*trans*-ACHC with a mean current of 146.3 nA (s.e. mean = ± 15.3, $n = 31$). For effects to be seen with the other members of the cyclohexane series long ejecting intervals (20-40 s) were required. There was a statistically significant difference in potency among the cyclohexane amino acid ($F_{4,108} = 4.45$; $P < 0.005$).

Antagonism of inhibition

The compounds, bicuculline and picrotoxin (but not strychnine), have been found to specifically antagonize the inhibitory action of GABA in the CNS (Curtis *et al.*, 1971; Barker & Nicoll, 1973). The inhibitory action of (±)-1,3-*cis*-ACPC most closely resembled that of GABA in potency and,

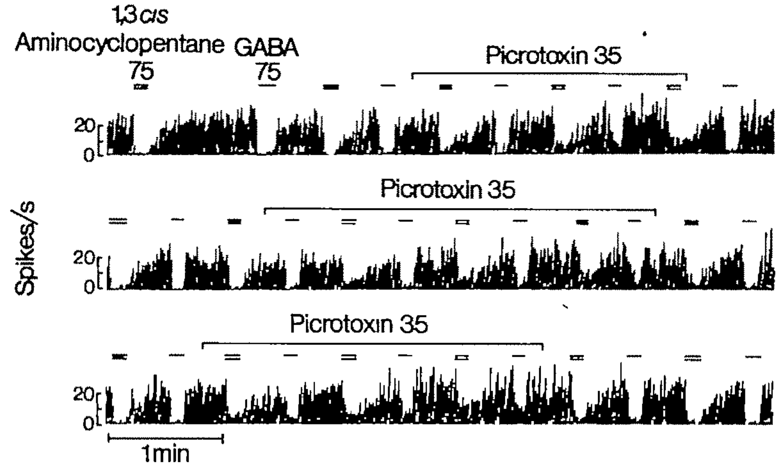

Figure 5 Antagonism of the inhibitory action of γ-aminobutyric acid (GABA) and (±)-1,3-*cis*-aminocyclopentane carboxylic acid by picrotoxin. The three traces are part of a continuous test on one cell and GABA application is indicated by single bars. Note the antagonism of inhibition despite the absence of a direct effect of picrotoxin on cellular activity.

therefore, was utilized as a representative analogue of GABA for comparing the effects of the selective pharmacological blocking agents.

Picrotoxin had a direct excitatory effect on the hippocampal cells tested at large currents (60-100 nA). However, using low currents with no direct effect (10-50 nA), effective antagonism of the inhibitory effect of both GABA and (±)-1,3-*cis*-ACPC was observed. This antagonism was demonstrated (Figure 5) in five out of six cells tested. Bicuculline also produced an increased rate of firing of hippocampal cells when large iontophoretic currents were used. Bicuculline, ejected with low iontophoretic currents which had no direct effect, reduced in an almost identical manner the efficacy of the (±)-1,3-*cis*-ACPC and GABA in three of the five cells studied. Strychnine was tested on six cells. Apart from its direct excitatory action, the agent did not significantly alter the response to either GABA or the (±)-1,3-*cis*-ACPC in any of the four cells tested.

Discussion

The results of the present study indicate that one receptor site mediating inhibition on rat hippocampal pyramidal cells accepts a molecule which has a spatial geometric separation of amino and carboxy functions of 4.6 Å or greater. Among the cyclopentane amino acids, the (±)-1,3-*trans* compound (separation = 4.7 Å) was the most effective in blocking spontaneous activity. Among the cyclohexane amino acids, the 1,3-*cis* compound (separation = 4.81 Å) was the most effective. Of the aliphatic natural amino acids, GABA was clearly the most effective inhibitor of spontaneous firing of hippocampal cells and the fully extended GABA molecule has a separation of carboxy and amino groups of 4.74 Å. These results indicate that the only conformational state of GABA capable of effectively activating the receptor site is the completely (or almost completely) extended molecule. In addition, these data suggest that glycine and β-alanine are not major inhibitory transmitters on hippocampal pyramidal cells. It should be emphasized that these results are applicable only to the cell type tested; i.e., the pyramidal cells of the CA_1 and CA_3 areas of rat hippocampal cortex. It is quite possible that other cell types in the central nervous system that are innervated by GABA-containing terminals may have a different type of receptor which requires a shorter spatial separation of amino and carboxyl functions. This may be the case where both GABA and glycine are postulated as being inhibitory transmitters (Curtis, Hösli, Johnston & Johnston, 1968) and the present approach would provide a means of investigating this interesting phenomenon.

The advantages inherent in the present experimental approach are derived from the use of compounds which: (1) have a well-defined, rigid spatial separation of amino and carboxyl groups; (2) have no other substituent groups which would significantly alter the chemical reactivity or electronic (charge density) characteristics of the amino or carboxyl groups, and are therefore almost identical to their corresponding aliphatic amino acids; and (3) represent a systematic serial variation of the spatial distance between amino and carboxy moieties. The first advantage is particularly relevant because the precise separation of functional groups for almost all compounds used as pharmacological agents is not known with certainty. Only in recent years, since the advent of the technique of nuclear magnetic resonance spectroscopy, has this facet of pharmacological agents been explored (Sutherland, 1973). The similarity of the cyclic amino acids to the natural amino acids in the electronic characteristics of their functional groups is particularly relevant to studies of drug-receptor relationships. The importance of the charge density of ions acting in a drug-receptor system was recently demonstrated experimentally by New & Richards (1972) using a hapten-antibody model. There, the affinity of antibody was a direct function of the charge density of the ammonium ion in the antigenic site. The similarity in charge density of the cyclohexane and cyclopentane amino acids to their corresponding naturally occurring aliphatic counterparts is illustrated by the almost identical pKa's of the corresponding sets of aliphatic and cyclic amino acids, (Maggiora, unpublished observations). By contrast, the difference in charge density of the carboxy ion which results from introduction of a single acetylenic bond is shown by the wide difference in pKa's of butyric acid ($CH_3-CH_2-CH_2-COOH$, pKa = 4.82) and 2-butynoic acid ($CH_3-C\equiv C-COOH$, pKa = 2.65). In this respect, it is pertinent that the compound, 4-aminotetrolic acid, employed by Beart *et al.* (1971), as a 'conformationally-restricted analogue of gamma-aminobutyric acid' would be expected to possess marked chemical differences from GABA.

The present results which indicate that the physiologically active conformation of GABA is the fully extended molecule may provide some insight into the physiochemical basis underlying drug receptor interactions. An aqueous solution of GABA at pH 7.0 consists of a mixed population of conformational states. The geometric separation of amino and carboxy groups may range from 2.4 Å in the 'folded' spatial conformer where the amino and carboxy ions are the most closely apposed to 4.8 Å in the 'fully extended' spatial conformer where the two functional groups are far apart. Previous studies (Segal *et al.*, 1973; Beart, Johnston & Uhr, 1972) using the cyclohexane amino acids indicate that the electrophysiological receptor for GABA may be different from the receptor responsible for GABA uptake. It is then conceivable that the two receptors use different conformational species of GABA. If this is the case, then it is plausible to assume that the physiological effect is terminated not by removal of the physiologically active molecule from the synaptic cleft, but rather by selective removal of a different population of spatial conformers. This is possible because of the extremely rapid equilibrium between the spatial conformations of the same molecule. A practical consideration derived from this concept is that pharmacological blockade of uptake should prolong the physiological effect, but the reverse situation, i.e., blockade of the receptor, should not inhibit uptake. A feasible means of testing this hypothesis of different receptors accepting different spatial conformations of the same molecule would be the use of the cyclopentane amino acid series in experiments testing their ability to affect GABA uptake.

It should be emphasized that this method of using spatial conformers to characterize an amino acid receptor can be extended to other amino acids. Indeed, this basic approach utilizing rigid spatial conformers has been extensively used to characterize acetylcholine muscarinic and nicotinic receptors (Beers & Reich, 1970). Future neurophysiological studies using rigid spatial conformers could add much to our knowledge of the nature and locus of action of neurotransmitters and, at the same time, provide insight into the molecular mechanisms responsible for the observed effect.

The authors wish to thank Dr L. Maggiora for the preparation of the compounds, and to acknowledge Drs F.E. Bloom and G.R. Siggins for their helpful comments on the manuscript; Mr L. Balentine for technical assistance, and Mr H. Poole, and Miss J. Peay for their invaluable assistance in the preparation of the manuscript.

Dr E. Smissman died in July 1974.

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(Received September 10, 1974.
Revised January 15, 1975)

Br. J. Pharmac. (1975), 54, 189-196

EVIDENCE FOR PROSTAGLANDIN MEDIATED PREJUNCTIONAL CONTROL OF RENAL SYMPATHETIC TRANSMITTER RELEASE AND VASCULAR TONE

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1 Prostaglandin E_2 dose-dependently and reversibly inhibited the noradrenaline overflow resulting from nerve stimulation of the rabbit kidney.

2 The magnitude of this inhibition varied inversely with the frequency of stimulation employed.

3 The prostaglandin synthesis inhibitors, indomethacin and meclofenamic acid, both increased the transmitter overflow resulting from renal nerve stimulation, suggesting that endogenous prostaglandin has a role in the regulation of transmitter release.

4 In the presence of indomethacin, the inhibitory effect of exogenous prostaglandin E_2 was enhanced.

5 The prostaglandin precursor, arachidonic acid, also caused a significant, dose-dependent and reversible inhibition of transmitter overflow. This inhibition became insignificant when arachidonic acid was applied in the presence of indomethacin, suggesting that the inhibition was mediated by newly formed prostaglandin rather than by arachidonic acid itself.

6 It is proposed that newly formed prostaglandin controls noradrenaline release primarily from inner cortical nerve endings, thereby maintaining juxtamedullary blood flow under periods of increased sympathetic nerve activity.

Introduction

The rabbit kidney has a high capacity for both synthesis (predominantly in the medulla) and degradation (in the renal cortex) of prostaglandins (Hamberg, 1969; Crowshaw, 1971; Larsson & Anggård, 1973). Prostaglandin E_2 is the most abundant renal prostaglandin and is a potent vasodilator (Lee, Crowshaw, Takman, Attrep & Gougoutas, 1967; Daniels, Hinman, Leach & Muirhead, 1967). Consequently, it has been postulated that this prostaglandin has a physiological role in blood flow regulation in the kidney (McGiff, Crowshaw, Terragno & Lonigro, 1970; Lonigro, Terragno, Malik & McGiff, 1973; McGiff & Itskovitz, 1973; Herbaczynska-Cedro & Vane, 1973, 1974; Larsson & Anggård, 1974; Needleman, Douglas, Jalsetik, Stoecklein & Johnson, 1974). It has in addition been shown that both renal nerve stimulation (RNS) and catecholamine administration increase the output of prostaglandins in the renal venous effluent of rabbits, prostaglandin E_2 being most abundant (Davis & Horton, 1972; Needleman *et al.*, 1974). In the light of previous evidence which suggests a role for prostaglandin E_2 in regulation of transmitter release from sympathetic nerve endings (Hedqvist, 1970, 1973, 1974a, b), it was decided to examine the possibility of such an interaction in the rabbit kidney. Previous experiments have shown that prostaglandin E_2 has an inhibitory effect on vascular responses of the rabbit kidney to RNS *in vitro* and *in situ* and that this inhibition is probably, in part, prejunctional (Frame, Hedqvist & Åström, 1974; Frame & Hedqvist, 1974).

The aim of the present study was to assess the capacity of prostaglandin E_2, prostaglandin precursor (arachidonic acid) and prostaglandin synthesis inhibitors (indomethacin, meclofenamic acid) to affect transmitter overflow resulting from RNS.

Methods

Forty rabbits weighing 2 to 3 kg were anaesthetized with 25% urethane, 7 ml/kg. The abdomen was opened along its midline and the left kidney, with its nervous and vascular supply, was dissected free from the surrounding tissue. The nerve was then carefully freed from the artery. After heparinization (1000 i.u./kg, i.v.) the vessels and ureter were cannulated, the nerve was cut and the kidney was flushed with 0.9% w/v NaCl solution (saline) containing 50 i.u. of heparin per ml. The preparation was then transferred to a perfusion chamber maintained at 37°C and was perfused with Tyrode solution (mM: NaCl 136.7, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.0, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, glucose 5.5, ascorbic acid 0.1) also at 37°C, containing 2% dextran and gassed with 5% CO_2 in O_2.

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The renal perfusion rate was kept constant at 10 ml/min and pressure was recorded on a Grass Model 5 polygraph using a Statham P 23 AC pressure transducer.

Noradrenaline (NA) stores were labelled by infusion of 25-50 μCi of tritiated NA in Tyrode solution over a period of about 20 minutes. The kidney was then perfused with isotope-free Tyrode solution for 30 min to remove surplus tritiated NA.

The nerve was placed on platinum electrodes and stimulated with a Grass S5 stimulator delivering 15 to 75 s trains of pulses (2-10 Hz, 2 ms, supramaximal voltage) at 10 min intervals. The total number of pulses applied per stimulation period was kept constant regardless of frequency. The perfusate was collected in 1 min fractions for 2 min prior to nerve stimulation, during stimulation and for 2 min post-stimulation. The tritium content of the perfusate was determined by counting 0.5 ml aliquots in a Packard liquid scintillation spectrometer with quenching monitored by internal standards. Twenty ml of a 3 : 7 ethanol-toluene mixture containing 4 g of 2-5 diphenyloxazole (PPO) and 0.1 g of 1-4 di (2-(4-methyl-5-phenyloxazolyl))benzene (POPOP) per litre of toluene was used as a counting medium for each sample.

The radioactivity of the perfusate samples was separated into that of intact NA and that of its metabolites by cation exchange column chromatography (Fredholm & Hedqvist, 1973). The recovery of authentic NA added to the samples and carried through the entirely chromatographic procedure was 73.3 ± 2.3% (mean ± s.e. mean, $n = 4$).

The following drugs were used: arachidonic acid, Sigma Chemicals, St. Louis, U.S.A.; [^{3}H]-(−)-noradrenaline, 7.2 Ci/mmol, The Radiochemical Centre, Amersham; indomethacin was a gift from Merck, Sharpe & Dohme, Rahway, N.J., U.S.A.; meclofenamic acid from Parke Davies, Pontypool, Wales, and prostaglandin E_2 from Dr J. Pike, Upjohn Co., Kalamazoo, Mich., U.S.A.

Arachidonic acid (99%, porcine liver) was further purified by silicic acid column chromatography shortly before use. All drugs were administered close-arterially to the kidney in Tyrode or saline solution.

Results

Stimulation of the renal nerves (2-10 Hz, 2 ms duration, supramaximal voltage) caused a marked increase in overflow of radioactivity from the kidney, which was accompanied by an increase in the perfusion pressure. During RNS, intact [^{3}H]-NA accounted for 81.3% of the radioactivity, compared to 14.4% during resting periods. In contrast, material not adsorbed to the column (mainly deaminated products) accounted for only 16.8% during RNS but 74.4% during resting periods. The content of [^{3}H]-normetanephrine, which made up 10% of the radioactivity during rest decreased to less than 2% during stimulation (Table 1).

Effect of exogenous prostaglandin E_2

It was consistently found that infusions of prostaglandin E_2 (1.8-9.1 x 10^{-7}M) 2 min prior to and during RNS, which did not alter the basal renal arterial pressure, decreased the overflow of transmitter in response to RNS in a dose-dependent manner (Figure 1). The degree of inhibition of [^{3}H]-NA overflow varied inversely

Table 1 Cation exchange column chromatography of radioactivity appearing in venous effluent of rabbit kidney previously loaded with [^{3}H]-noradrenaline.

Sample	*No. of expts.*	*'Acid metabolites'*	*Noradrenaline*	*Normetanephrine*
Stimulated	7	16.8 ± 2.3	81.3 ± 2.2	1.3 ± 0.4
Resting	5	74.4 ± 2.3	14.4 ± 1.1	10.0 ± 1.5

Perfusate samples withdrawn before and during nerve stimulation. Chromatographic values presented as relative distribution (per cent) of recovered 'acid metabolites' (mainly deaminated products), intact noradrenaline and normetanephrine, and given as means ± s.e. mean.

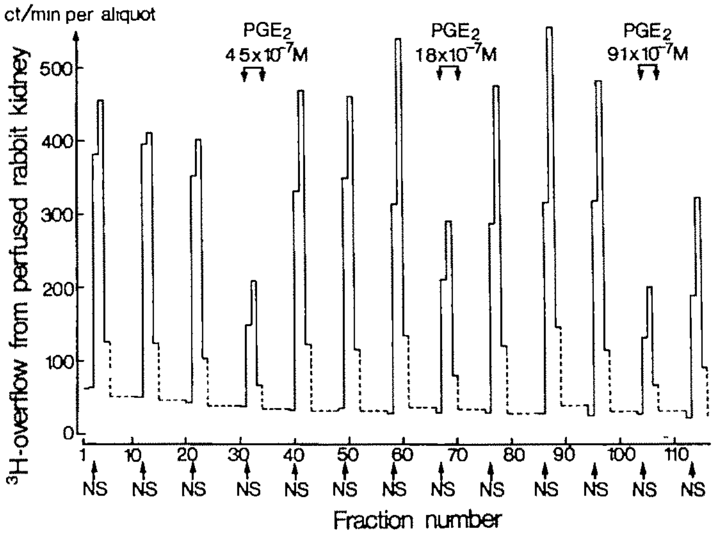

Figure 1 Isolated, perfused kidney of the rabbit, preloaded with [^{3}H]-noradrenaline. Effect of prostaglandin E$_2$ (PGE$_2$) on overflow of tracer resulting from renal nerve stimulation (NS), 150 pulses at 5 Hz. Time in min = fraction numbers. Dotted lines represent uncollected fractions.

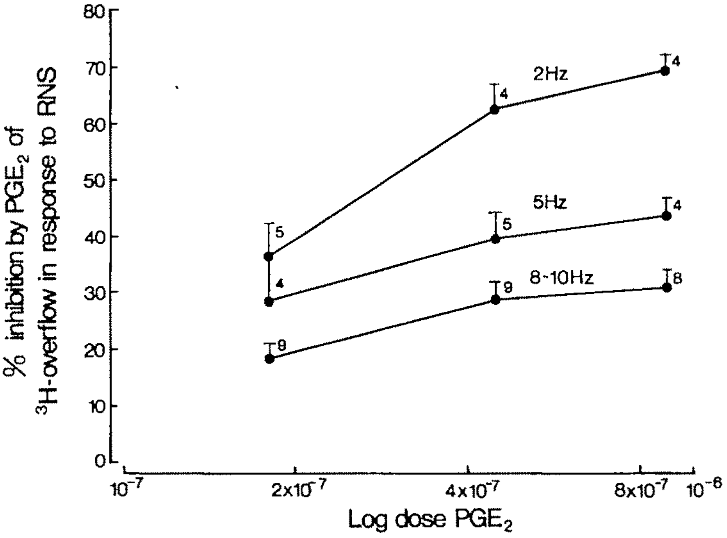

Figure 2 Inhibitory effect of prostaglandin E$_2$ (PGE$_2$) on transmitter overflow resulting from renal nerve stimulation (RNS), 150 pulses, 2-10 Hz. Vertical bars show s.e. mean. Figures at the points = number of observations.

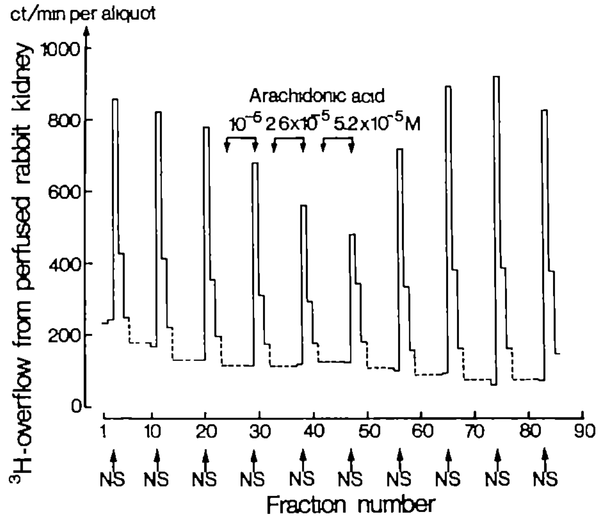

Figure 3 Effect of cumulative doses of arachidonic acid on transmitter overflow resulting from renal nerve stimulation (NS), 150 pulses at 5 Hz.

with the stimulation frequency employed (Figure 2). The vascular responses to RNS were similarly inhibited and there was partial or complete recovery from both [^{3}H]-NA overflow and vascular-inhibition 10 min after ending the infusion of prostaglandin E_2.

Effect of arachidonic acid

When arachidonic acid (2.6 x 10^{-5}M) was infused 5 min prior to and during RNS, transmitter overflow was inhibited by 35.3% (see Table 2). On the addition of cumulative doses of arachidonic acid, a dose-dependent inhibition was observed (Figure 3). Concomitantly there was a corresponding reduction of the vascular response to RNS. In most of the experiments, a slight reduction in perfusion pressure was observed throughout the duration of the infusion. Partial or complete recovery from the inhibition usually occurred within 10-20 min of ending the arachidonic acid infusion, although in some experiments the transmitter overflow remained depressed.

Effect of prostaglandin synthesis inhibitors

Indomethacin was used to block the synthesis of endogenous prostaglandins (Vane, 1971) and thus to evaluate the possible role of the latter in adrenergic transmission in the kidney.

At a stimulation frequency of 5 Hz (150 pulses), infusion of indomethacin (4.4 x 10^{-5}M) sometimes caused a slight increase in the resting perfusion pressure (up to 5 mmHg) and either no effect or a slight decrease in the effector responses to RNS. The effect on transmitter overflow, however, was much more marked. The overflow of [^{3}H]-NA during RNS increased significantly 8 min after the start of an infusion of indomethacin and plateaued after about 15 min of infusion. The maximal increase of [^{3}H]-NA overflow in response to RNS was 41.5 ± 7.0% (mean ± s.e. mean, $n = 13$). This value was significant at the $P < 0.001$ level according to Student's t-test for paired varieties. After ending the infusion, the transmitter overflow fell off approaching the preinfusion level within 10 to 20 min. However, in some experiments the transmitter overflow remained at an elevated level throughout post-infusion controls (Figure 4).

In three additional experiments, meclofenamic acid, (5 x 10^{-5}M) which is also a potent inhibitor of prostaglandin synthesis (Gryglewski & Vane, 1972), was used instead of indomethacin. The increase in [^{3}H]-NA overflow in response to RNS produced by infusion of meclofenamic acid was 23.3 ± 3.5% ($P < 0.05$). The drug caused qualitatively the same effects as indomethacin, although it was less potent on an equimolar basis.

Effect of prostaglandin E_2 and arachidonic acid application during indomethacin treatment

During indomethacin treatment, prostaglandin E_2 was still effective in producing an inhibition of [^{3}H]-NA overflow in response to RNS. The

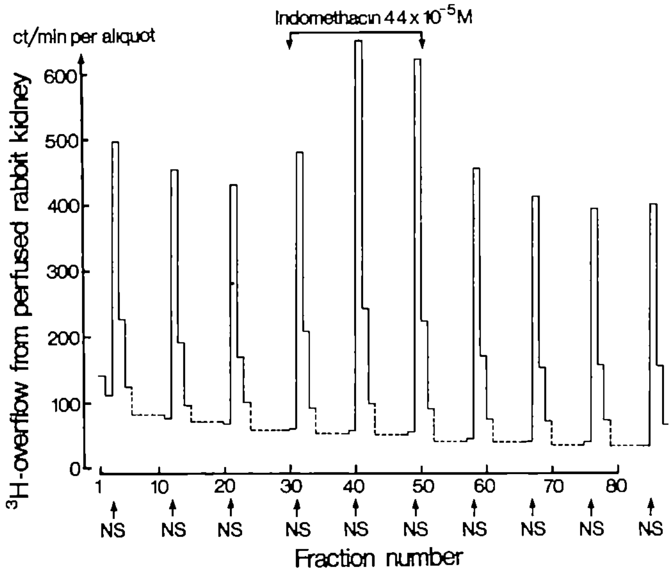

Figure 4 Effect of indomethacin on transmitter overflow resulting from renal nerve stimulation (NS), 150 pulses at 5 Hz.

inhibition produced was, in fact, significantly greater ($P < 0.02$) than that produced by prostaglandin E_2 alone, the values being 61.3% for indomethacin plus prostaglandin E_2 and 39.6% for prostaglandin E_2 alone (Table 2).

The inhibitory effect of arachidonic acid, unlike that of prostaglandin E_2, was significantly reduced ($P < 0.001$) from 35.3% for arachidonic acid alone to 10.6% for arachidonic acid in the presence of indomethacin. This would suggest that the inhibitory effect was primarily that of newly formed prostaglandin rather than that of the arachidonic acid itself (Figure 5, Table 2).

Discussion

It was previously shown that prostaglandin E_2 inhibits renal vascular responses to RNS and, to a lesser extent, exogenous NA in the dog and rabbit kidney (Lonigro *et al.*, 1973; Frame *et al.*, 1974). Those results merited the suggestion of a primary prejunctional action of prostaglandin E_2, as observed in other tissues (cf. Hedqvist, 1973).

The overflow of NA from an adrenergically innervated organ can be monitored by determining the efflux of [^{3}H]-NA previously taken up by the nerves (Hertting & Axelrod, 1961). Since in the

Table 2 Percentage change of transmitter overflow to RNS (5 Hz, 150 pulses) by prostaglandin E_2 (PGE_2) (4.5×10^{-7}M), arachidonic acid (2.6×10^{-5}M) and indomethacin (4.4×10^{-5}M), and by prostaglandin E_2 and arachidonic acid in the presence of indomethacin.

Administered compound	*Pretreatment* *None*	 *Indomethacin*	*Difference* *None — indomethacin*
PGE_2	−39.6 ± 4.5(5) $P < 0.001$	−61.3 ± 4.6(3) $P < 0.01$	21.7 ± 6.4 $P < 0.02$
Arachidonic acid	−35.3 ± 4.0(5) $P < 0.001$	−10.6 ± 4.4(5) $P < 0.1$ (NS)	24.7 ± 5.9 $P < 0.001$
Indomethacin	+41.5 ± 7.0(13) $P < 0.001$	—	—

Mean values ± s.e. mean, figures within brackets = number of experiments. Statistical analysis according to Student's *t*-test.

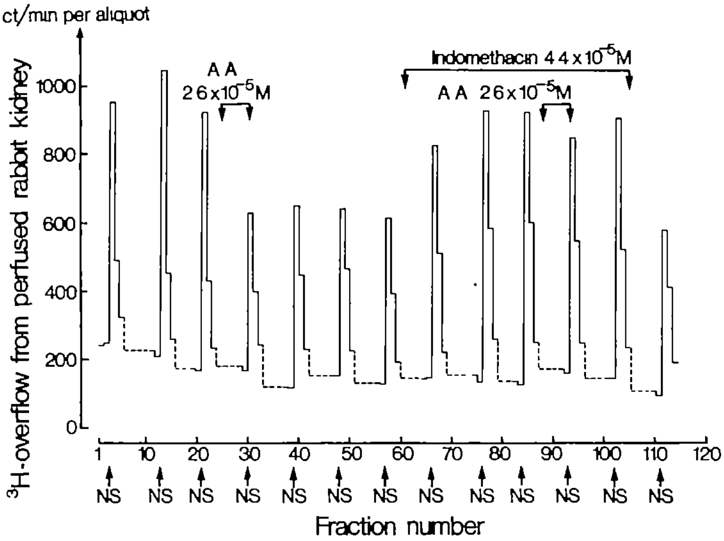

Figure 5 Effect of arachidonic acid (AA) alone and of arachidonic acid in the presence of indomethacin on transmitter overflow resulting from renal nerve stimulation (NS, 150 pulses at 5 Hz)

present study the majority of the radioactivity overflowing in response to RNS was represented by intact [^{3}H]-NA, total radioactivity was considered an appropriate indicator of endogenous NA overflow.

It is apparent from this study that transmitter overflow resulting from RNS is dose-dependently and reversibly inhibited by exogenous prostaglandin E_2 and can also be modified by altering endogenous prostaglandin levels. Judging from experiments on the cat spleen and the guinea-pig vas deferens and heart (Hedqvist, 1970, 1974a; Bhagat, Dhalla, Ginn, LaMontagne & Montier, 1972), where prostaglandin E_2 does not alter the disposition of released NA, it is likely that the observed inhibition of transmitter overflow represents an action directly on the process of excitation-secretion coupling in the nerve terminals.

In examining the inhibitory or 'braking' effect of exogenous prostaglandin E_2 on transmitter release, the control transmitter overflow to RNS acted as a guide to the effect of prostaglandin E_2. However, it could not be regarded as a true control since, at least in the dog and rabbit kidney, RNS causes release of prostaglandin E_2 which should also provide some braking action (Dunham & Zimmermann, 1970; Davis & Horton, 1972; McGiff, Crowshaw, Terragno, Malik & Lonigro, 1972). In an attempt to reduce the interference of endogenous prostaglandins, experiments were carried out in which prostaglandin synthesis inhibitors were administered to the kidney. The primary effect of the synthesis inhibitors, indomethacin and meclofenamic acid (Vane, 1971; 1973), was to elevate transmitter overflow, which is to be expected if an endogenous braking-mechanism has been partially or wholly removed. Moreover, in harmony with previous observations on the guinea-pig vas deferens (Hedqvist, 1974a), a given dose of prostaglandin E_2 showed an increased ability to inhibit transmitter overflow during indomethacin infusion. This increased potency may also be attributable to removal of a local braking mechanism, assuming that administered prostaglandin E_2 normally has to compete with endogenous prostaglandin for active sites in the nerve terminals.

In a series of experiments the inhibitory effect of prostaglandin E_2 on transmitter release was studied at different stimulation frequencies. Generally the inhibition produced by a given dose varied inversely with the stimulation frequency employed. This would seemingly suggest an endogenous prostaglandin-mediated braking mechanism which operates more readily at a low impulse activity than at a high. This is in good agreement with results obtained in the rabbit heart (Junstad & Wennmalm, 1973) where the sympathetic transmitter-release-mechanism became less sensitive to exogenous prostaglandin E_2 as the frequency of stimulation was increased from 2-10 Hz. The release of *endogenous* prostaglandin during sympathetic nerve stimulation of the dog kidney and the rabbit heart shows no apparent linear correlation with either the frequency or the transmitter overflow (Dunham & Zimmerman, 1970; Junstad & Wennmalm, 1973). Thus an increase in stimulation frequency from 2-10 Hz only approximately doubled the efflux of prostaglandin E_2-like material per unit time whilst the transmitter overflow increased tenfold. It would seem, therefore, that the endogenous transmitter braking-mechanism is capable of compensating for changes in impulse activity but that its effectiveness is limited both by the amount of prostaglandin releasable and by the sensitivity of the NA-release process to prostaglandin.

The series of experiments in which the prostaglandin precursor arachidonic acid was used, was carried out in an attempt to show that newly synthesized prostaglandins have access to sympathetic junctions. Infusion of arachidonic acid produced a marked inhibitory effect on both transmitter overflow and vascular responses to RNS. This effect was mainly due to the formation of prostaglandins since the effect was very much reduced or abolished when arachidonic acid was infused in the presence of indomethacin. A small residual effect of the arachidonic acid is not unexpected, since indomethacin in doses similar to that used in the present study has been shown to inhibit markedly but not abolish the conversion of arachidonic acid to prostaglandin E_2 in rabbit kidney and in rat kidney homogenates (Larsson & Anggård, 1974; Fredholm, Hedqvist & Larsson, unpublished observations).

In discussing a role for prostaglandins as regulators of transmitter release at renal sympathetic nerve endings, the assumption is made that there is a ready source of prostaglandins in the vicinity of the cortical nerve endings. The biosynthesis of prostaglandins, however, has been shown to be ten times greater in the medulla than in the cortex (Larsson & Anggård, 1973). It has, nevertheless, been emphasized by these authors that cortical synthesis is large enough to be of significance. The renal cortex, in addition to its biosynthetic capacity, possesses an abundant amount of prostaglandin degrading enzyme, prostaglandin dehydrogenase (Anggård, Larsson & Samuelsson, 1971). It is probable, however, that endogenous cortical prostaglandin which escapes degradation may have access to the cortical nerve endings. This possibility is inhanced by the fact that prostaglandin E_2 infused into the present system, although going directly to the cortex, produced an effect on RNS. An alternative suggestion is that medullary prostaglandins may reach the juxtamedullary arterioles via the vasa recta (Larsson & Anggård, 1973).

Although both the juxtamedullary and outer cortical arterioles have adrenergic innervation, it is interesting that during RNS or induction of stress in rabbits, the outer cortex becomes ischaemic whilst juxtamedullary cortical blood flow persists (Trueta, Barclay, Daniel, Franklin & Pritchard, 1947). Moreover, the prostaglandin synthesis capacity of the cortex increases towards the medulla and stimulation of prostaglandin synthesis using arachidonic acid increases juxtamedullary blood flow in the rabbit (Larsson & Anggård, 1973; 1974). It seems, therefore, that prostaglandins are primarily available in the juxtamedullary zone to inhibit sympathetic arteriolar tone. It is apparent from the present study that this dilatation or prevention of vasoconstriction is, at least in part, attributable to inhibition of transmitter release, i.e. a prejunctional action on adrenergic neuro-effector transmission. However, part of the effect may be post-junctionally located at the effector cell level since prostaglandin E_2 to some extent inhibits vascular responses to NA in the dog and rabbit kidney (McGiff *et al.* 1972; Frame *et al.*, 1974) and newly formed prostaglandin increases juxtamedullary blood flow in the dog kidney under *in vitro* conditions (Itskovitz, Terragno & McGiff, 1974).

This study was supported by grants from the Swedish Medical Research Council, project no. 04X-4342, and from Magnus Bergvalls Stiftelse. M.F. wishes to thank the Swedish Institute for a guest scholarship and the Wellcome Trust for a travel grant. Reprint requests to M.F. in Edinburgh.

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(Received November 14, 1974.
Revised February 18, 1975)

Br. J. Pharmac. (1975), 54, 197-204

THE EFFECTS OF PROSTAGLANDINS ON GUINEA-PIG ISOLATED INTESTINE AND THEIR POSSIBLE CONTRIBUTION TO MUSCLE ACTIVITY AND TONE

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1 Prostaglandins $F_{1\alpha}$ and $F_{2\alpha}$ caused contraction of the longitudinal muscle of both guinea-pig isolated ileum and colon, apparently by acting directly on the muscle and on cholinergic nerves. They had little effect on ileal circular muscle.

2 Prostaglandins E_1 and E_2 caused contraction of the longitudinal muscle of guinea-pig isolated colon, apparently by acting directly on the muscle and on excitatory nerves which are non-cholinergic. Prostaglandin E_1 seems more effective than E_2 in stimulating these nerves.

3 It seems likely that prostaglandin release *in vitro* maintains the tone of the longitudinal muscle of guinea-pig colon, whereas release of a prostaglandin E compound inhibits circular muscle tone.

Introduction

Guinea-pig ileum contains prostaglandin E_2- and $F_{2\alpha}$-like substances (Ambache, Brummer, Rose & Whiting, 1966). The tissue releases prostaglandin E_2-like activity on incubation in Krebs solution, and the quantities are increased by electrical field stimulation (Botting & Salzmann, 1974). Both prostaglandin E and F compounds contract longitudinal muscle of guinea-pig isolated ileum (Bergström, Eliasson, Euler & Sjöval, 1959; Weeks, Schultz & Brown, 1968; Bennett, Eley & Scholes, 1968) and colon (Karim, 1966; Bennett & Fleshler, 1969a; Bennett & Posner, 1971). Prostaglandin F compounds contract the circular muscle of guinea-pig colon (Fleshler & Bennett, 1969), but in contrast prostaglandin E compounds inhibit intestinal circular muscle (Bennett *et al.*, 1968; Fleshler & Bennett, 1969). On the basis of this type of evidence many have thought that prostaglandins might play a role in regulating gastrointestinal activity.

It has been suggested that prostaglandin release maintains the tone in rabbit isolated jejunum (Ferreira, Herman & Vane, 1972), and may play a similar role in the longitudinal muscle of guinea-pig isolated ileum (Davison, Ramwell & Willis, 1972; Botting & Saltzmann, 1974). Prostaglandins have also been implicated in contractions mediated by cholinergic nerves in this tissue (Ehrenpreis, Greenberg & Belman, 1973; Kadlec, Mašek & Šeferna, 1974; Bennett, Eley & Stockley, 1975). Thus, the contribution of prostaglandins to intestinal activity might depend on the types and relative amounts released, their site of release, and the intrinsic nerve activity. The aim of the present work was to determine, in those parts of the guinea-pig intestine not previously studied, the effects and mechanism of action of prostaglandins on muscle strips, and to evaluate their contribution to muscle tone by the use of drugs which inhibit prostaglandin synthesis or action.

Methods

The ileum and colon were removed from freshly killed guinea-pigs, and 1-2 cm long segments of mid-ileum, and of colon approximately 10 cm from the anus, were used to study the longitudinal muscle. Spirals were cut for studies of the circular muscle. The preparations were suspended in Krebs solution at 37°C bubbled with 5% CO_2 in O_2 under a load of 0.5-1 g, and changes in length were registered by isotonic transducers and pen recorders. Electrical field stimulation consisting of alternating square wave pulses of 1 ms duration (generated by linked stimulators 6051 and 6053, Scientific and Research Instruments Ltd.) was applied between platinum electrodes near the top and bottom of the organ bath giving a voltage drop of 3.4 V/cm measured in Krebs solution. The formula of the Krebs solution was (g/l): NaCl 7.1; $CaCl_2.6H_2O$ 0.55; KCl 0.35; KH_2PO_4 0.16; $MgSO_4.7H_2O$ 0.29; $NaHCO_3$ 2.1; dextrose 1.0.

Drugs

The following drugs were used: 1-acetyl-2-(8-chloro-10,11-dihydrodibenz(b,f)(1,4)oxazepine-10-carbonyl) hydrazine (SC-19220), acetylcholine perchlorate, acetylsalicylic acid, histamine acid phosphate, (–)-hyoscine hydrobromide, indomethacin, nicotine hydrogen tartrate, polyphloretin phosphate (PPP), potassium chloride, prostaglandins E_1, E_2, $F_{1\alpha}$, and $F_{2\alpha}$ tromethamine salt, and tetrodotoxin. All concentrations of salts are expressed as base or free acid. The prostaglandins were dissolved in ethanol (0.1 ml per mg) and diluted with 0.9 ml sodium carbonate solution 0.2 mg/ml. Indomethacin 1 mg or aspirin 10 mg was dissolved in 1 ml 0.9% w/v NaCl by adding solid sodium carbonate to pH7.

Results

Effects and mechanism of action of prostaglandins $F_{1\alpha}$ and $F_{2\alpha}$ on the ileum

In 14 longitudinal muscle preparations from 12 animals prostaglandin $F_{1\alpha}$ (200-300 ng/ml) and $F_{2\alpha}$ (20-225 ng/ml) caused contractions. Tetrodotoxin (TTX; 0.1 or 0.2 μg/ml) prevented contractions to nicotine and reduced those to prostaglandins in both of the experiments with prostaglandin $F_{1\alpha}$ (average 16% reduction), and in 10 of 11 experiments with $F_{2\alpha}$ (overall reduction 32 ± 13% (s.e.), $P < 0.01$). Prostaglandin E_2, which has been studied previously, was used for comparison; TTX reduced its effect by 52 ± 6%, $n = 5$, $P < 0.01$. The muscle tone and contractions to acetylcholine (ACh) were not significantly altered by TTX (ACh reduced by 5 ± 10%, $n = 11$). Hyoscine (0.1 or 0.2 μg/ml), given before TTX, or after it had been washed out and contractions to nicotine had returned, prevented responses to ACh and nicotine, and consistently reduced those to prostaglandin $F_{1\alpha}$ (67 ± 12%, $n = 4$, $P < 0.05$), $F_{2\alpha}$ (60 ± 6%, $n = 9$, $P < 0.005$), and E_2 (52 ± 7, $n = 5$, $P < 0.02$) (Figure 1). The addition of TTX in the presence of hyoscine (4 experiments) and *vice-versa* (2 experiments) usually had little further effect on the responses to prostaglandin $F_{2\alpha}$.

Prostaglandin $F_{1\alpha}$ and $F_{2\alpha}$ had little or no effect on circular muscle of the guinea-pig ileum. In one experiment prostaglandin $F_{2\alpha}$ 1.1 μg/ml caused a small contraction, but $F_{2\alpha}$ 0.3-1.1 μg/ml or $F_{1\alpha}$ 0.4-4 μg/ml had no effect in two others. In another strip prostaglandin $F_{1\alpha}$ or $F_{2\alpha}$ (both 0.5 μg/ml) caused small increases in the contraction to KCl 2 mg/ml. Neither prostaglandin $F_{2\alpha}$ (0.1-1 μg/ml, three experiments) nor $F_{1\alpha}$ (0.1-1 μg/ml, one experiment), in contact with the tissue for 5-15 min, altered submaximal contractions elicited by electrical stimulation at 1-4 Hz. Because of this lack of activity the prostaglandin F compounds were not examined further in circular ileal muscle.

Effects and mechanisms of action of prostaglandins $F_{2\alpha}$ and E_2 in colonic longitudinal muscle

In 26 experiments on tissue from 24 animals prostaglandin $F_{2\alpha}$ (75-300 ng/ml) or E_2 (20-40 ng/ml) in contact with the tissue for 2-2.5 min caused a slow contraction which was occasionally preceded by a brief relaxation. After washing prostaglandin from the bath the muscle relaxed slowly, taking 10-15 min to reach the baseline.

Hyoscine (0.1 or 0.2 μg/ml) prevented contractions to ACh and sometimes lowered the muscle tone. Nicotine-induced contractions were variably affected (prevented, reduced, no effect or increased in two, one, one and one experiments respectively, overall reduction 40 ± 26%), and nicotine-induced relaxations were enhanced in two experiments and revealed in one. Contractions to prostaglandin $F_{2\alpha}$ were mainly reduced (35 ± 16%, $n = 10$, $P < 0.05$), but responses were increased in two of these tissues whose tone was lowered by hyoscine (perhaps the increase due to lowering of tone overshadowed the effect of hyoscine). Although there was a tendency for hyoscine to reduce contractions to prostaglandin E_2, the overall effect was not statistically significant (overall reduction 19 ± 10%, $P > 0.1$). There was a reduction in 15 out of 18 experiments and an increase in three tissues; in two of these three instances the tone was reduced by hyoscine. However, if the latter two experiments are excluded because of the fall in tone, there was a significant reduction of the effect of prostaglandin E_2 by hyoscine (31 ± 7%, $n = 16$, $P < 0.01$).

TTX (0.1 to 0.4 μg/ml) sometimes reduced the tone of colonic longitudinal muscle. It always greatly reduced or prevented the response (contraction, relaxation or relaxation followed by contraction) to nicotine, but had no significant effect on contractions to ACh (reduction of 5 ± 5%, $n = 14$). It reduced contractions to prostaglandin $F_{2\alpha}$ in nine out of ten experiments (overall reduction of 39 ± 10%, $P < 0.01$) and to E_2 in 11 out of 14 experiments (overall reduction of 24 ± 9%, $P < 0.01$). In two experiments contractions to prostaglandin E_2 were unaffected, and in one experiment in which TTX greatly lowered the muscle tone contractions to both prostaglandin $F_{2\alpha}$ and E_2 were increased.

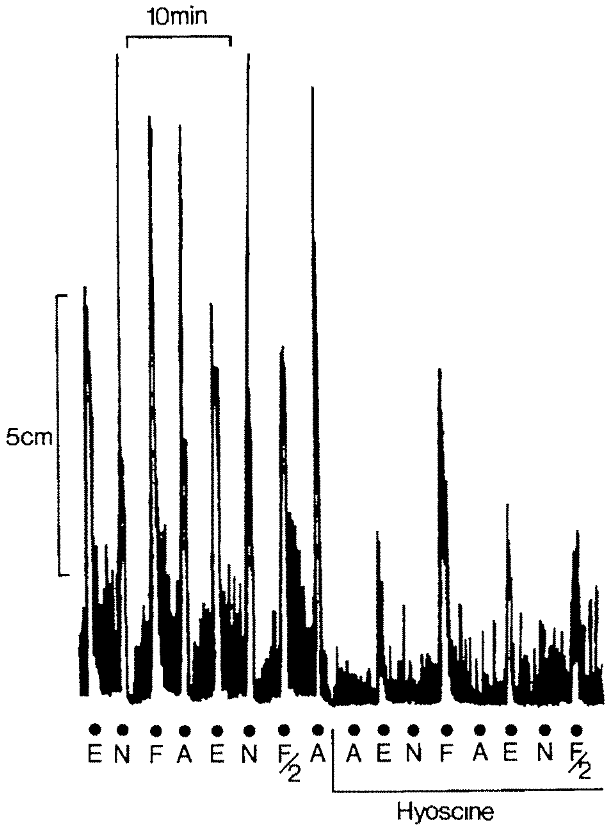

Figure 1 Hyoscine (0.1 μg/ml) prevented contractions of the longitudinal muscle of guinea-pig isolated ileum to acetylcholine (A, 20 ng/ml) and nicotine (N, 3 μg/ml), and reduced responses to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (F, 0.1 μg/ml) and E_2 (E, 10 ng/ml).

Comparison of prostaglandins E_1 and E_2 in colonic longitudinal muscle

Since the reduction with TTX of contractions to prostaglandin E_2 was less than the average of 60% reported for E_1 in this tissue (Bennett & Fleshler, 1969a), E_1 and E_2 were compared in 11 further experiments. The reduction of prostaglandin E_1 by TTX (0.1-0.4 μg/ml) alone (8 experiments) or in the presence of hyoscine (0.1-0.2 μg/ml, 3 experiments) was greater than of E_2 (54 ± 7% and 26 ± 6% respectively, $P < 0.0025$; Figure 2). There was no difference between the effect of hyoscine on prostaglandin E_1 and E_2 ($P > 0.7$) or the reduction of E_2 by TTX or hyoscine ($P > 0.5$), but the reduction of E_1 by TTX was greater than by hyoscine ($P < 0.05$).

The possible relation of prostaglandins to muscle tone

Ileal circular muscle The inhibitory effects of prostaglandins E_1 and E_2 demonstrated previously on this tissue (Bennett *et al.*, 1968) have been substantiated in further experiments which show that E_2 (0.1-1 μg/ml) inhibited electrically induced contractions of circular muscle. Aspirin (10-40 μg/ml) or indomethacin (1 μg/ml; Figure 3) (four experiments each) caused a rise (usually small) in tone, with initiation or enhancement of spontaneous contractions. Furthermore, they potentiated contractions produced by electrical field stimulation at 0.1-8 Hz. The degree of potentiation varied with the stimulating frequency, being most pronounced at low frequencies even though all responses remained submaximal. At 0.1 Hz contractions were initiated or enhanced, but this was difficult to measure because of increased spontaneous activity. There was a significant increase of 301 ± 110% at 1 or 2 Hz ($n = 14$; $P < 0.0005$), but the increase at 4 and 8 Hz (20 ± 10%) was not significant ($n = 8$; $0.05 < P < 0.1$) and in one strip the contraction at 8 Hz was reduced by 27% (possibly due to the particularly marked rise in tone caused by indo-

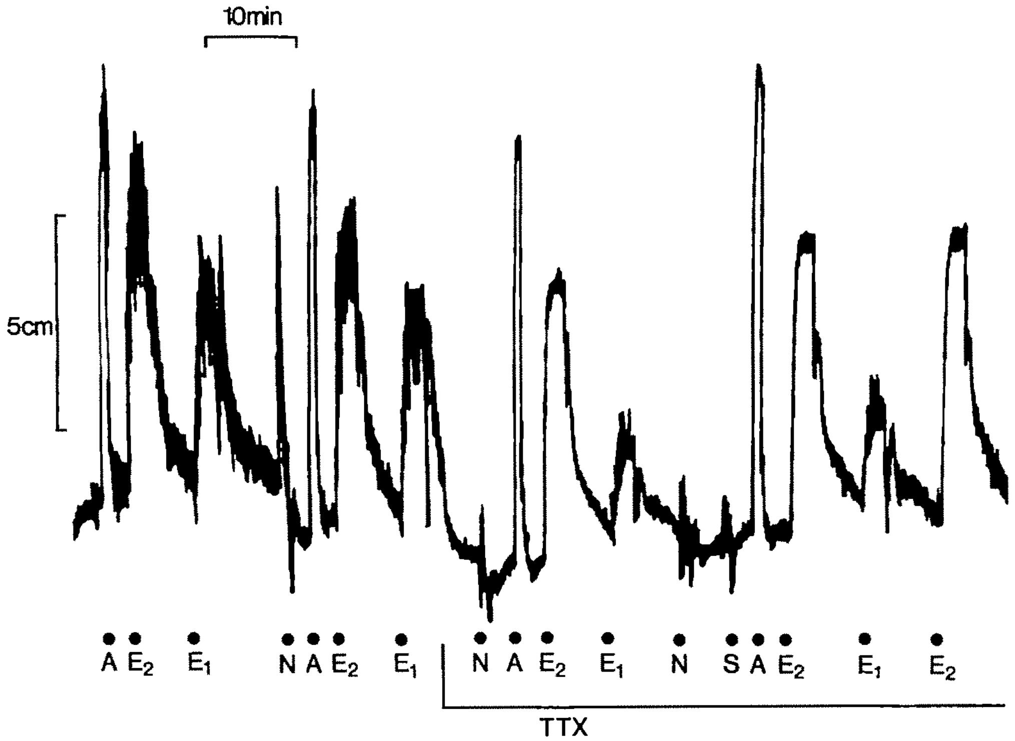

Figure 2 Longitudinal muscle of guinea-pig isolated colon. Tetrodotoxin (TTX, 0.4 μg/ml) prevented contractions to nicotine (N, 5 μg/ml), greatly reduced those to prostaglandin E_1 (E_1, 80 ng/ml), and slightly reduced those to E_2 (E_2, 10 ng/ml). Responses to acetylcholine (A, 20 ng/ml) were unaffected. S, saline 0.4 ml.

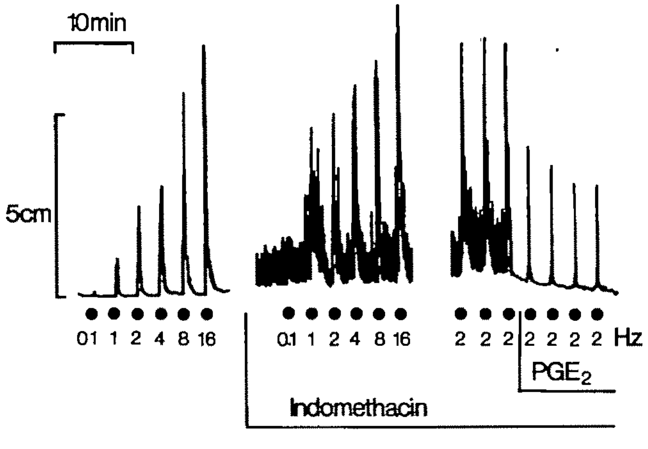

Figure 3 Guinea-pig ileal circular muscle responded to electrical excitation (1 ms pulses, 20 s trains) with contractions which were graded with the stimulating frequency. Indomethacin (1 μg/ml) initiated rhythmical activity, slightly raised the tone, and enhanced the electrically-induced contractions. Prostaglandin E_2 (PGE_2, 0.1 μg/ml) reversed all of these effects. The intervals in the trace were 60 min (incubation with indomethacin) and 75 min (before recording the effect of prostaglandin E_2).

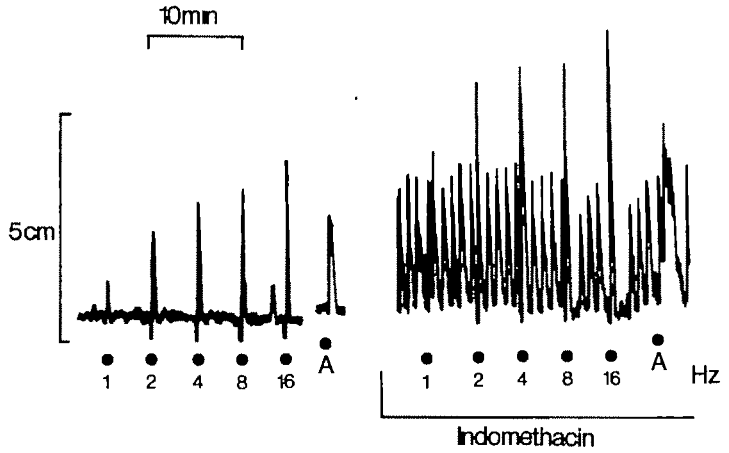

Figure 4 Electrically-induced and spontaneous contractions of the circular muscle of guinea-pig isolated colon were increased by indomethacin (1 μg/ml). The responses to acetylcholine (A, 5 μg/ml) were also increased in this and in two out of three other experiments with indomethacin, but they were unaffected by aspirin (two studies) despite larger responses to electrical stimulation.

methacin). The effects of aspirin and indomethacin were reversed by prostaglandin E_2 (0.1-1 μg/ml) as illustrated in Figure 3. Polyphloretin phosphate (PPP, 1-50 μg/ml; two experiments) or SC-19220 (10 μg/ml; two experiments) did not antagonize the inhibition of electrically stimulated contractions by prostaglandin E_2, nor did they mimic the stimulant effects of aspirin and indomethacin on muscle tone. However, these substances do not block the inhibitory effect of prostaglandin E compounds on the circular muscle (Bennett & Posner, 1971).

Colonic circular muscle Aspirin (50-100 μg/ml, five experiments) raised the tone and increased or initiated spontaneous activity in this tissue, but the effects were generally less pronounced than in the ileum. Indomethacin (1-4 μg/ml) raised the tone and increased rhythmic activity in two preparations and increased the tone in one, but failed to alter spontaneous activity in two others. Electrical stimulation at 0.5-32 Hz caused relaxation followed by an after-contraction. Both prostaglandin synthetase inhibitors regularly enhanced the after-contraction to an extent which depended on the stimulating frequency (Figure 4); responses to 1 and 16 Hz were increased respectively by 171 ± 29% and 40 ± 31% over control by aspirin, and by 136 ± 40% and 30 ± 14% by indomethacin (five experiments each). Hyoscine (5 μg/ml, six experiments) reduced the after-contractions by 25 ± 5%, and after-contractions elicited in the presence of hyoscine (5 μg/ml) were unaffected by aspirin or indomethacin (two experiments each).

Colonic longitudinal muscle Aspirin (20 or 100 μg/ml, four experiments) or indomethacin (1 or 2 μg/ml, four experiments) lowered the tone of the preparations, taking 30-90 min to reach a steady level. The gradual relaxation was accompanied by an increase in amplitude of the spontaneous activity and enhancement of submaximal contractions to ACh (10-100 ng/ml), prostaglandin E_2 (5-20 ng/ml), and contractile components of responses to electrical excitation at 0.1-8 Hz (Figure 5). The effect on contractions is expressed quantitatively in Table 1. The potentiation of contractions by indomethacin was less for ACh than for electrical stimulation ($P < 0.01$) but this effect did not reach statistical significance with aspirin ($0.05 < P < 0.1$). After the addition of prostaglandin E_2 (2 or 4 ng/ml) the tone and responses to electrical stimulation were not significantly different from the controls before addition of synthetase inhibitor. However, responses to ACh were significantly smaller (by 30 ± 9%, $P < 0.05$).

Discussion

Effects of prostaglandins on longitudinal and circular muscle preparations

The responses conform in general to the pattern of contraction of both muscle layers with prostaglandin F compounds, but longitudinal muscle contraction and circular muscle inhibition with the prostaglandin E compounds (Bennett & Fleshler, 1970). In the longitudinal muscle of guinea-pig

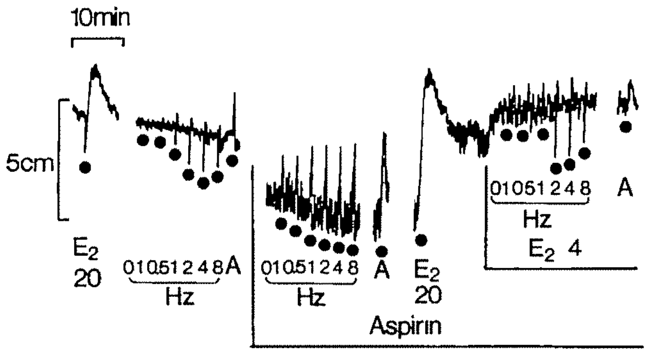

Figure 5 Guinea-pig colonic longitudinal muscle gave biphasic responses (relaxation then contraction) to prostaglandin E_2 (E_2, 20 ng/ml) and electrical stimulation (1 ms pulses; 10 s trains), and contracted to acetylcholine (A, 10 ng/ml). In the presence of aspirin (100 μg/ml for 1 h) the tone fell and spontaneous and acetylcholine-induced contractions increased. The responses to electrical stimulation and prostaglandin E_2 were converted to contractions which were bigger than the previous contractile component of the biphasic responses. After washing out prostaglandin E_2 (20 ng/ml) in the continued presence of aspirin, the tone remained elevated at almost the control level. Subsequent addition of a lower concentration of prostaglandin E_2 (E_2, 4 ng/ml) restored responses to electrical excitation and acetylcholine to approximately their control values.

ileum prostaglandins $F_{1\alpha}$ and $F_{2\alpha}$ behaved much like E_1 and E_2 in previous studies (Bennett *et al.*, 1968; Harry, 1968) in that the responses were reduced by TTX and hyoscine. It therefore seems that the prostaglandin E and F compounds have two sites of action, the cholinergic nerves and the muscle cells.

The similar pA_2 values for prostaglandins E_2 and $F_{2\alpha}$ with the antagonist SC-19220 (Bennett & Posner, 1971) are consistent with the possibility that both act at the same receptor sites. Although the reduction of contractions to prostaglandin $F_{2\alpha}$ by hyoscine seemed greater than that by TTX, the difference was not significant ($0.05 < P < 0.1$).

In the circular muscle strips of guinea-pig ileum prostaglandins $F_{1\alpha}$ and $F_{2\alpha}$ had little effect on their own or on responses to electrical stimulation, suggesting that prostaglandins of the F-series are unlikely to play a role in regulating the motility of this muscle layer.

In the longitudinal muscle of guinea-pig colon prostaglandins $F_{2\alpha}$ and E_2 caused slow contractions, occasionally preceded by a brief relaxation. With both prostaglandins a neural contribution was indicated since TTX reduced the responses. The reduction of contractions to prostaglandin $F_{2\alpha}$ by hyoscine suggests that cholinergic nerves may be involved, but the picture with prostaglandin E_2 is less clear. A significant reduction by hyoscine was seen only if two experiments were excluded from the calculations. In these two instances the responses to prostaglandin E_2 actually increased after hyoscine, but this might have been due to the lowering of muscle tone. In previous experiments (Bennett & Fleshler, 1969a) evidence was obtained that prostaglandin E_1 stimulated only non-cholinergic excitatory nerves. It seems that prostaglandin E_1 is more effective than E_2 in stimulating these nerves, since we have shown that TTX, but not hyoscine,

Table 1 Guinea-pig longitudinal distal colonic muscle. Enhancement of contractions (% increase over control ± s.e.) to electrical stimulation, acetylcholine (ACh) or prostaglandin E_2 (PGE_2) by aspirin (20 or 100 μg/ml) or indomethacin (1 or 2 μg/ml), and reversal of the effect by prostaglandin E_2 (2 or 4 ng/ml).

	Aspirin	*Indomethacin*	*PGE_2 plus aspirin or indomethacin*
1,2 and 4 Hz	194 ± 44 (12) **	120 ± 18 (12) **	17 ± 10 (18) NS
ACh 10-100 ng/ml	80 ± 33 (4) NS	46 ± 16 (4) *	−30 ± 9 (6) *
PGE_2 5-20 ng/ml	140 ± 73 (4) *	97 ± 18 (4) **	

The numbers in brackets represent the numbers of experiments. (NS, $P > 0.05$; * $P < 0.05$; ** $P < 0.01$).

caused a significantly greater reduction of the effect of E_1 than of E_2. The presence of non-cholinergic excitatory nerves was confirmed by our experiments with nicotine. As reported previously with nicotine and dimethylphenylpiperazinium (Bennett & Fleshler, 1969b, 1970; Bennett, 1971), TTX virtually prevented the contractions to nicotine, whereas hyoscine had a variable effect on the responses and complete blockade of contractions occurred in only one experiment. The only difference in the present study was that responses to nicotine sometimes consisted of relaxations or biphasic changes and not just contractions. The reason for the difference is not known.

Prostaglandins and intestinal muscle tone

The results with the prostaglandin synthesis inhibitors suggest that an E-type prostaglandin might be released continuously to reduce or prevent the generation of tone and spontaneous activity in the circular muscle of the ileum and colon. Stimulation by these substances of spontaneous and electrically evoked contractions was more pronounced in the ileum than in the colon. In both regions prostaglandin E compounds are potent inhibitors of circular muscle activity, but only colonic circular muscle is sensitive to the excitatory effect of F compounds. Thus, if both types of prostaglandins are normally generated simultaneously in the colon and exert mutually antagonistic effects on the circular muscle, inhibition of their synthesis might have little overall effect. The prostaglandin antagonists PPP and SC-19220 did not mimic the response to the synthesis inhibitors in the circular muscle of the ileum, presumably because they do not antagonize the inhibitory effect of prostaglandins E_1 and E_2 on alimentary circular muscle (Bennett & Posner, 1971).

Potentiation by aspirin or indomethacin of electrically induced after-contractions in the colonic circular muscle was prevented by hyoscine, suggesting the involvement of a cholinergic mechanism. Prostaglandin F compounds do not seem to stimulate the cholinergic nerves in this tissue (Fleshler & Bennett, 1969), but removal of inhibition by an E-type prostaglandin might be responsible. Perhaps prostaglandin inhibits the release of ACh from the cholinergic nerves, as has been suggested in the rabbit heart (Wennmalm & Hedqvist, 1971). Indomethacin would then increase the activity of the cholinergic system. A similar mechanism might occur with cholinergic nerve stimulation in ileal circular muscle.

On the other hand, Botting & Salzmann (1974) concluded that in the longitudinal muscle of guinea-pig ileum prostaglandin was unlikely to inhibit ACh release, because ACh output was the same or reduced by indomethacin. The relatively high concentrations of indomethacin (10 and 20 μg/ml) used by Botting & Salzmann (1974) might have depressed ACh release non-selectively, thus masking a potentiation due to removal of prostaglandin. However, indomethacin reduced responses to electrical stimulation of this tissue in other studies, and added prostaglandin E_2 potentiated responses to cholinergic nerve stimulation (Ehrenpreis *et al.*, 1973; Kadlec *et al.*, 1974; Bennett *et al.*, 1975).

There is circumstantial evidence that prostaglandins contribute to the tone of the longitudinal muscle of guinea-pig colon. Aspirin or indomethacin lowered the tone and this was restored by low concentrations of prostaglandin E_2. A non-selective depressant action of the prostaglandin synthetase inhibitors seemed unlikely, since responses to ACh and electrical stimulation actually increased. However, the increases, which were probably due mainly to the lower tone, might have overshadowed a non-selective depression. When the tone was restored by prostaglandin E_2 in the presence of indomethacin, responses to ACh were smaller than normal. Perhaps indomethacin depresses the responses to ACh but increases the effect of electrical stimulation, so that there is no net change in the response to cholinergic nerve activation.

Taken together with the data of Davison *et al.* (1972) and Botting & Salzmann (1974), our results suggest that prostaglandins contribute to the tone of the longitudinal muscle of guinea-pig isolated ileum and colon. In contrast, prostaglandin E_2 might tend to keep intestinal circular muscle in a relaxed state. It remains to be determined whether prostaglandins exert these effects only when released in 'dying' isolated preparations, or whether they play a physiological role in controlling gut motility.

We thank Dr J.E. Pike, Upjohn Company Ltd., U.S.A. for prostaglandins, Dr B. Högberg, Leo Research Laboratories, Sweden for polyphloretin phosphate, and the Wellcome Trust for support (H.L.S.).

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(Received September 10, 1974.
Revised January 7, 1975)

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THE RESPONSES OF THE ISOLATED, BLOOD-PERFUSED SPLEEN OF THE DOG TO ANGIOTENSIN, OXYTOCIN AND VASOPRESSIN

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1 The responses of the smooth muscle of the capsule and blood vessels of the isolated, blood-perfused spleen of the dog to angiotensin, oxytocin and vasopressin have been investigated and compared to the actions of the catecholamines, adrenaline and noradrenaline.

2 Increasing doses of each of the three polypeptides cause graded increases in splenic vascular resistance and reductions in spleen volume.

3 Doses of the polypeptides which evoked increases in splenic vascular resistance not significantly different from increases produced by chosen doses of each catecholamine caused significantly smaller reductions in spleen volume.

4 The time-course of action of the polypeptides on the splenic vascular smooth muscle is different since the time to 50% recovery from vasopressin is highly significantly longer than that for equieffective doses of either angiotensin or oxytocin.

5 Phenoxybenzamine, in a dose which almost blocked the actions of the catecholamines, increased the responses of the vascular and capsular smooth muscle to oxytocin, vasopressin and angiotensin. This increase was not observed with another α-adrenoceptor blocking agent, phentolamine.

6 The significant species variation in the responses of the smooth muscle of the spleen to polypeptides and catecholamines are discussed and the results are considered in the context of the possible physiological roles of the polypeptides in haemorrhage.

Introduction

The actions of the catecholamines, adrenaline and noradrenaline on the splenic capsular and vascular smooth muscle have been established in the dog (Davies, Gamble & Withrington, 1968a,b; 1973), cat (Greenway & Stark, 1970) and in man (Ayers, Davies & Withrington, 1970; 1972). However, although the responses to the polypeptides angiotensin, vasopressin and oxytocin have been investigated in the cat spleen (Greenway & Stark, 1970) and in the human isolated spleen (Ayers *et al.*, 1970; 1972) their actions have not been fully documented in the dog spleen. It is of importance to ascertain the range of smooth muscle responses to these substances so that an evaluation may be made of their possible cardiovascular role in situations when circulating levels are elevated.

In a previous publication, Davies, Robinson & Withrington (1969) analysed the responses of the smooth muscle of the dog's spleen to adrenergic nerve stimulation and to catecholamines after the administration of phenoxybenzamine. In order to demonstrate the continued functional viability of the splenic smooth muscle after adrenoceptor blockade, splenic contractions were elicited by the smooth muscle stimulant angiotensin. However, it was observed that, when the responses to catecholamines and sympathetic nerve stimulation were diminished or abolished, the splenic vasoconstrictor and capsular responses to angiotensin were slightly increased. In the present paper we have specifically studied this observation and confirmed the increased responses to angiotensin; in addition, experiments were designed to answer two questions. firstly, whether these increased responses were specific to this polypeptide, and secondly, whether they were related to some property of phenoxybenzamine other than that due to its α-adrenoceptor blocking action. For this purpose we have tested the splenic responses to two other polypeptides, vasopressin and oxytocin before and after phenoxybenzamine and, in addition, tested all three polypeptides before and

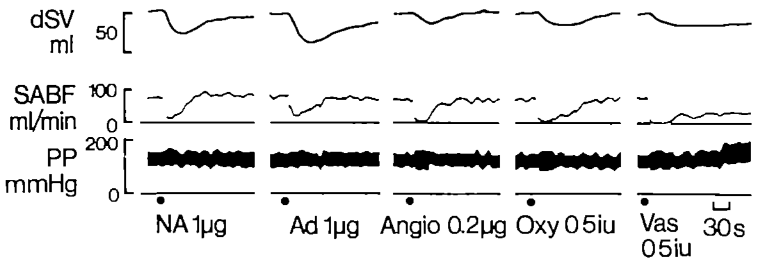

Figure 1 Changes in spleen volume (dSV), splenic arterial blood flow (SABF) and perfusion pressure (PP) in response to close arterial injections of noradrenaline 1 μg (NA), adrenaline 1 μg (Ad), angiotensin 0.2 μg (Angio), oxytocin 0.5 iu (Oxy) and vasopressin 0.5 iu (Vas) in the isolated perfused spleen of the dog.

after inducing adrenoceptor blockade with phentolamine.

Methods

Experiments were performed on 17 pairs of dogs (weights 8.1 to 19 kg) anaesthetized with an intravenous injection of 2-5 ml of 2.5% methohexitone sodium (Brietal, Lilly) followed by 5 ml/kg of a mixture of 1% chloralose (α-chloralose, Kühlmann, Paris) and 10% urethane (BDH) dissolved in 0.9% w/v NaCl solution (saline) and filtered.

Preparation of the spleen for perfusion was exactly as described in detail by Davies & Withrington (1968). Splenic arterial blood flow, phasic arterial perfusion pressure, splenic venous pressure and changes in spleen volume were recorded on a direct writing Beckman Type 'R' Dynograph.

Phasic perfusion pressure was recorded with a flexible polythene catheter from a side arm central to the rotameter with a Statham high pressure transducer (P23Gb). The system was at least as good as the present experimental system using a transducer (Type 4-327-0003, Consolidated Electrodynamics division of Bell & Howell) which has an undamped natural frequency of over 110 Hz with an estimated amplitude distortion of less then 5% up to 30 Hz. Mean perfusion pressure was, therefore, taken as the diastolic pressure plus one third of the pulse pressure. Splenic vascular resistance was then calculated as mean splenic arterial perfusion pressure/mean splenic arterial blood flow. Changes in splenic venous pressure, other than the transient increases accompanying the onset of capsular contraction, were negligible and not included in the calculations of changes in splenic vascular resistance. The postganglionic nerves to the spleen were dissected away from the artery and placed on platinum electrodes with the cathode distal. The electrode leads were connected to a stimulator and pulse counter so that a set number of pulses (50 V, 0.5 ms) could be delivered at a known frequency.

All drugs were administered into the rubber tubing, proximal to the cannulated splenic artery. Phenoxybenzamine and phentolamine were administered slowly over a period of 30 seconds. During the period of injection and for subsequent 30 s the venous blood draining the spleen was collected and discarded. This procedure restricted the action of the blocking drugs to the spleen and ensured that little entered the systemic circulation of the donor dog.

The following drugs were used: angiotensin (Hypertensin, Ciba), (−)-adrenaline (McCarthys); (−)-noradrenaline (Winthrop); oxytocin (Syntocin, Sandoz), vasopressin (Pitressin, Parke Davis & Co.); phenoxybenzamine (Dibenyline, SKF); and phentolamine (Rogitine, Ciba).

Results

In the dog spleen, perfused with blood at constant pressure, close-arterial injections of angiotensin, oxytocin and vasopressin caused a reduction in both spleen volume and splenic arterial blood flow. These responses are illustrated in Figure 1 where angiotensin (0.2 μg) and oxytocin (0.5 iu) both caused a reduction in arterial flow from 68 to 3 ml/min and vasopressin (0.5 iu) a reduction from 72 to 2 ml/minute. These reductions in flow represent increases in splenic vascular resistance of 2,190% for angiotensin and oxytocin and 3,520% for vasopressin. Small contractions of the splenic capsule accompanied these vascular effects, the concomitant reductions in volume in response to angiotensin, oxytocin and vasopressin being 12, 16 and 15 ml respectively. In contrast, the two catecholamines, adrenaline (1.0 μg) and nora-

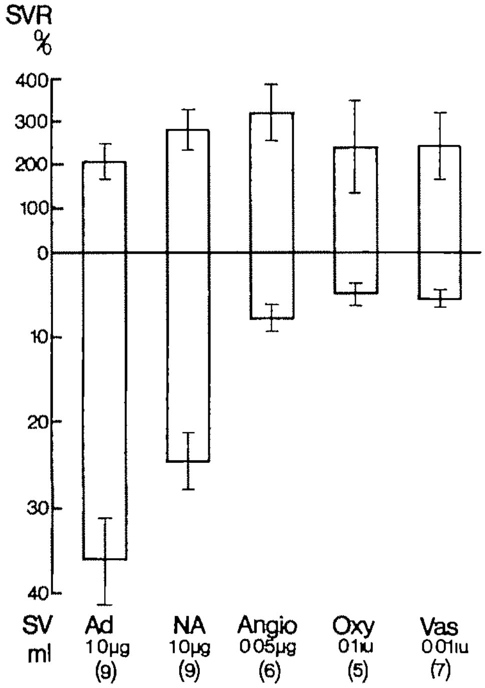

Figure 2 Mean percentage increase in splenic vascular resistance (SVR) and mean reduction in spleen volume (SV) produced by close arterial injection of adrenaline 1.0 μg (Ad), noradrenaline 1.0 μg (NA), angiotensin 0.05 μg (Angio) oxytocin 0.1 iu (Oxy) and vasopressin 0.01 iu (Vas). Vertical lines represent s.e. mean and figures in brackets refer to number of observations.

drenaline (1.0 μg), both caused small increases in splenic vascular resistance of 314% and 450%, but were associated with much larger reductions in splenic volume of 36 and 37 ml respectively.

The essential difference between the responses of the splenic vascular and capsular smooth muscle to these five substances is shown in Figure 2, which contains data from 10 experiments in each of which at least four of the five substances were tested. In each experiment, a dose of each substance was selected which on close-arterial injection would produce approximately the same vascular response. In this series of experiments the mean increase in splenic vascular resistance to adrenaline (1.0 μg), noradrenaline (1.0 μg), angiotensin (0.05 μg), oxytocin (0.1 iu) and vasopressin (0.01 iu) were 214 ± 41%, 293 ± 48%, 337 ± 68%, 258 ± 109% and 255 ± 81% respectively. Statistical analysis revealed no significant difference between the vascular responses to adrenaline and noradrenaline ($P > 0.1$) or between adrenaline and any of the three polypeptides, angiotensin ($P > 0.05$), oxytocin ($P > 0.30$) and vasopressin ($P > 0.30$). However, the concomitant responses of the splenic capsule to the five substances were strikingly different, the mean reductions in spleen volume being 35.9 ± 5.1 ml, 24.4 ± 3.3 ml, 7.3 ± 1.6 ml, 4.6 ± 1.3 ml and 5.2 ± 1.1 ml to adrenaline, noradrenaline, angiotensin, oxytocin and vasopressin respectively. These experiments confirm the previous observations (Davies *et al.*, 1973) that the capsular response to adrenaline is significantly greater than to the same dose of noradrenaline ($P = 0.05$) and in addition show that the reduction in spleen volume in response to the three polypeptides are all highly significantly smaller ($P < 0.0005$). The results clearly demonstrate a difference in the reactivity of the splenic vascular and capsular smooth muscle to catecholamines and polypeptides.

The ratio of potency of these five substances on the splenic capsular and vascular smooth muscle will depend on the dose level. It is not practicable, in the isolated spleen perfused at constant pressure, to establish the complete dose-response curves for capsular and vascular smooth muscle to all three polypeptides in the same preparation. The long duration of action of each polypeptide sets a limit to the number of injections and, in addition, the potent vasoconstrictor properties of each polypeptide prevents their access to capsular smooth muscle at high dose levels. The mean responses of the capsular and vascular smooth muscle to three different dose levels of each polypeptide are illustrated in Figure 3. The doses were chosen to cover the range of vascular effects from just above threshold to almost complete vasoconstriction, i.e. cessation of splenic arterial blood flow. It is clear that the responses of the capsule and blood vessels to angiotensin, oxytocin and vasopressin are dose-dependent but that there is no clear dose-dependent separation of responses as previously reported with the catecholamines. An indication of the relative potencies of the five substances on the splenic vascular smooth muscle may be made at the single dose level for each shown in Figure 2. In this test adrenaline (1.0 μg), noradrenaline (1.0 μg), oxytocin (0.2 μg), angiotensin (0.05 μg) and vasopressin (0.02 μg) were equieffective in increasing splenic vascular resistance (1.0 iu oxytocin or vasopressin is equivalent to approximately 2.0 μg, Goodman & Gilman, 1970).

It is apparent (Figure 1) that the polypeptides have different time-courses of action on the splenic vascular smooth muscle. In 10 tests in which doses of angiotensin, oxytocin and vasopressin produced similar maximum increases in splenic vascular resistance, the mean time to 50% recovery was similar for angiotensin and oxytocin (51.8 ± 5.9 s and 67.7 ± 6.2 s respectively,

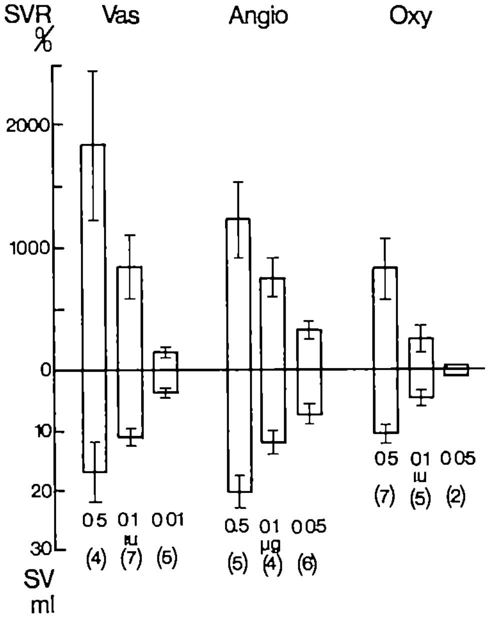

Figure 3 Mean percentage increases in splenic vascular resistance (SVR) and mean reductions in spleen volume (SV) produced by close-arterial injections of graded doses of vasopressin (Vas), angiotensin (Angio) and oxytocin (Oxy). Vertical lines represent s.e. mean and the figures in brackets refer to the number of observations.

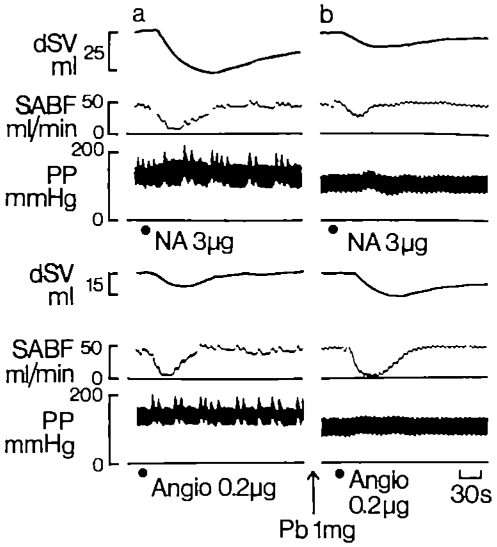

Figure 4 Changes in spleen volume (dSV), splenic arterial blood flow (SABF) and perfusion pressure (PP) in response to close-arterial injections of noradrenaline 3 μg (NA) and angiotensin 0.2 μg (Angio) before (a) and after (b) phenoxybenzamine 1 mg (Pb).

$P = 0.05$). The vascular response to vasopressin was, however, of much longer duration, with a mean time to 50% recovery of 269 ± 46.6 seconds. This is highly significantly longer than for equieffective doses of either angiotensin or oxytocin ($P < 0.0005$).

The effects of phenoxybenzamine

In each experiment, control responses of both the capsular and vascular smooth muscle were obtained to at least four of six standard tests. These were close-arterial injection of adrenaline, noradrenaline, angiotensin, oxytocin and vasopressin, and electrical stimulation of the splenic sympathetic nerves at 3 Hz. Doses of the three polypeptides were selected which produced marked but submaximal effects. Phenoxybenzamine was then given by the close-arterial route, in a dose (1-3 mg) which almost abolished the splenic responses to adrenaline, noradrenaline and splenic nerve stimulation when the standard tests were repeated.

This procedure was carried out in 11 experiments with angiotensin and part of the results of one experiment is shown in Figure 4, in which the responses to noradrenaline (3 μg) were a decrease in flow, equivalent to an increase in splenic vascular resistance of 354% and a reduction in spleen volume of 29 ml, which were reduced after phenoxybenzamine (1 mg) to 55% and 9 ml respectively. However, in the same experiment, the responses to angiotensin (0.2 μg), which were initially an increase in splenic vascular resistance of 955% and a reduction in spleen volume of 10 ml, were increased after phenoxybenzamine to 1690% and 15 ml respectively. In addition, the vascular response was considerably prolonged. Consideration of the results of all 11 experiments show that the splenic capsular response to angiotensin (0.025-0.2 μg) was increased after phenoxybenzamine in seven tests with no change or a slight reduction in the remaining four. The mean reduction in spleen volume in all 11 tests was 11.2 (± 1.3) ml before, and 14.0 (± 1.7) ml after phenoxybenzamine. The concomitant increases in splenic vascular resistance produced by angiotensin were increased in ten tests after phenoxybenzamine and showed no change in the remaining one. In eight of the ten tests, potentiation was apparent as a greater increase in splenic vascular resistance, the mean increases being 589% (± 133) before and 1018% (± 172) after phenoxybenzamine. In the other two tests, the control vascular response to angiotensin was near maximal

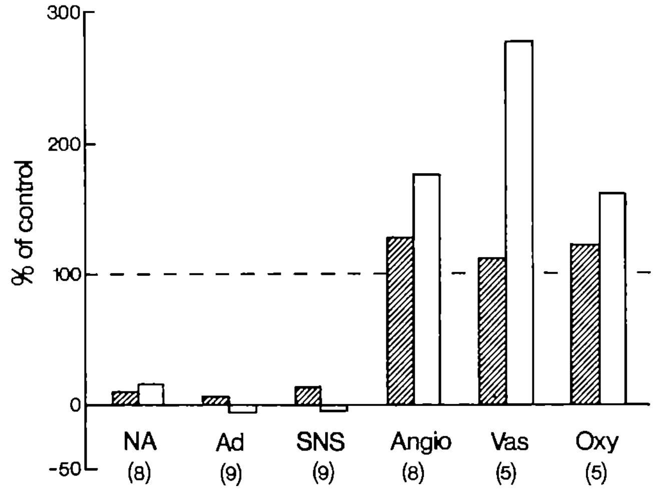

Figure 5 The effects of phenoxybenzamine on changes in splenic vascular resistance (open columns) and reductions in spleen volume (hatched columns) in response to sympathetic nerve stimulation at 3 Hz (SNS), and close-arterial injections of noradrenaline (NA), adrenaline (Ad), angiotensin (Angio), vasopressin (Vas) and oxytocin (Oxy). In each test the response after phenoxybenzamine is expressed as a percentage of the control response before phenoxybenzamine (= 100%). Each column represents the mean of a number of tests which is indicated in brackets.

and the increased response after phenoxybenzamine was apparent as a greatly increased duration. Paired analysis of the responses in each experiment to the same dose of angiotensin, before and after phenoxybenzamine, showed that the increases in both the capsular and vascular responses are significantly greater than zero ($P < 0.0005$ and $P < 0.01$ respectively).

Vasopressin (0.02-0.5 iu) was tested by close arterial injection in 5 experiments and the mean reduction in spleen volume was 18.8 ml (± 8.2). In four of these experiments the capsular response to the same dose of vasopressin was increased after phenoxybenzamine but the capsular response was unchanged in one experiment. The mean reduction in volume to the same doses of vasopressin after phenoxybenzamine was 20.6 ml (± 7.2). The splenic vascular responses to these doses of vasopressin were also potentiated in 4 of the 5 tests and unchanged in one. In one experiment the potentiation was more apparent as a considerably prolonged response. The mean increase in splenic vascular resistance in the four tests was 364% (± 114) before, and 987% (± 266) after phenoxybenzamine. Paired analysis of the capsular and vascular responses to the same dose of vasopressin in each experiment revealed that the increases in responses after phenoxybenzamine were significantly greater than zero ($P < 0.025$ and $P < 0.0125$ respectively).

Oxytocin (0.01-0.5 iu) was injected by the close arterial route in seven experiments. In four of these there was an increase in the capsular contraction to oxytocin after phenoxybenzamine, in two there was no change and in one a small reduction. The mean reduction in spleen volume in all seven tests was 11.4 ml (± 1.5) and 13.6 ml (± 2.4) after phenoxybenzamine. The splenic vascular response was potentiated in all seven tests. In five of these the increase in splenic vascular resistance produced by oxytocin was 462% (± 114) initially and 728% (± 212) after phenoxybenzamine. In the remaining two tests, the near maximal vascular responses were prolonged. Paired analysis showed that the increases in both capsular and vascular responses are significantly greater than zero ($P < 0.01$ in both cases).

These differences between the splenic capsular and vascular responses to the three polypeptides, which are all increased after phenoxybenzamine, and the responses to sympathetic nerve stimulation and to the catecholamines, which are all reduced or even reversed, are apparent in Figure 5, where the control responses before phenoxybenzamine are represented as 100% and the subsequent responses after phenoxybenzamine are expressed as a percentage of the control.

Effects of phentolamine

In three experiments control responses to angiotensin, adrenaline, noradrenaline and sympathetic nerve stimulation were obtained prior to the close-arterial administration of a dose of phentolamine (1.0 mg) which blocked the responses to sympathetic nerve stimulation and the injected catecholamines. In these experiments the mean initial responses to angiotensin (0.2 μg) were a reduction in spleen volume of 5.7 ml (± 2.4) and an increase in splenic vascular resistance of 343% (± 191). After the administration of phentolamine the mean responses were not significantly different being 6.0 ml (± 2.3) and 350% (± 226) respectively. Similarly, in single experiments with oxytocin and vasopressin, the administration of phentolamine had no effect on the responses of either the splenic capsular and vascular smooth muscle to either of the polypeptides.

Discussion

The three polypeptides, angiotensin, vasopressin and oxytocin have been shown to possess potent actions on the smooth muscle of the capsule and blood vessels of the dog's spleen. Qualitatively, the predominant action of each polypeptide is on the smooth muscle of the splenic blood vessels causing profound vasoconstriction, although all three substances provoke, in addition, a contraction of the smooth muscle forming the splenic capsule. Angiotensin has been shown to produce similar responses in the cat spleen (Hertting & Suko, 1966; Greenway & Stark, 1970) but vasopressin, in the same preparation caused vasoconstriction with little or no capsular contraction. Experiments on the human isolated perfused spleen (Ayers *et al.*, 1972) revealed that whilst both angiotensin and vasopressin caused vasoconstriction, oxytocin was devoid of any vasoconstrictor properties. There were no accompanying reductions in spleen volume. A considerable species variation therefore exists in the responses of the two splenic smooth muscle systems to these naturally occurring polypeptides (Davies & Withrington, 1973). Furthermore, it is apparent that the relative sensitivity of the two smooth muscle systems to the polypeptides is different from their sensitivity to catecholamines (Davies *et al.*, 1973) since doses of angiotensin, vasopressin or oxytocin which caused constrictions of the splenic vascular bed not significantly different from adrenaline or noradrenaline, provoked significantly smaller reductions in spleen volume than either catecholamine.

The duration of action of angiotensin and oxytocin on the splenic vascular bed is similar but both have a significantly shorter half-life of action than vasopressin. The half-lives of circulating arginine and lysine vasopressin in the dog have been found by radioimmunoassay techniques to be 6.0 and 5.4 min respectively (Biro, Forsling, Martin & Wilmott, 1972). Preliminary experiments (Davies, Forsling & Withrington, unpublished observations) have indicated that the half-life of oxytocin in the dog is slightly shorter. No comparable information exists about the half-life of angiotensin in the dog but superfusion techniques (Ferriera & Vane, 1967) suggest a half-life of 20 seconds. These techniques measure the disappearance of polypeptides from the general circulation but give no indication of their relative disappearance by passage through spleen. Possibly the longer recovery time for the splenic smooth muscle from administered vasopressin is related to a more complete uptake from the blood. No quantitative information is available to verify this point.

The mode of action of angiotensin on smooth muscle has been explained in a number of ways. A central action (Aars & Akre, 1968) may be excluded in the present experiments since the preparation was isolated. Similarly, since the splenic smooth muscle responses were not reduced by doses of phenoxybenzamine, which partially blocked the responses to sympathetic nerve stimulation and injected catecholamines, the possibility that the action of angiotensin on splenic smooth muscle is mediated by the release of stored catecholamines in the nerve endings (Distler, Liebau & Wolff, 1965) may be eliminated. This is in agreement with the observations of Thoenen, Hurlimann & Haefely (1965) who showed that the splenic responses to angiotensin were not reduced by phenoxybenzamine and also with the conclusions of Hertting & Suko (1966) who demonstrated that the responses to angiotensin and vasopressin were unaffected by either chronic sympathectomy or pre-treatment with reserpine. Furthermore, in the present experiments when the responses to stimulation of adrenoceptors were partially blocked by phenoxybenzamine the splenic smooth muscle responses to angiotensin, vasopressin and oxytocin were increased. Paired analysis revealed that the increase in capsular and vascular responses to all three polypeptides after phenoxybenzamine was significant. In each experiment the splenic arterial blood flow before and after phenoxybenzamine was the same so that the increased responses did not arise simply from increases in the arterial concentration of each polypeptide, secondary to reductions in perfusion. However, it is possible that phenoxybenzamine improves the access of polypeptides to the receptor sites by altering the microcirculation within the spleen. Phenoxybenzamine has a number of well-defined actions including the blockade of α-adrenoceptors, blockade of uptake processes into both nerve and muscle and in addition causes an increase in the release of noradrenaline from nerve endings in response to sympathetic nerve stimulation. All these actions appear at different dose levels of phenoxybenzamine (Enero, Langer, Rothlin & Stefano, 1972) and makes the interpretation of the increased responses observed in the present experiments difficult. However, since in the same experiments the responses to the directly acting polypeptides oxytocin and vasopressin were increased a further action of phenoxybenzamine may be to potentiate the responses of smooth muscle to nonadrenergic stimuli. Furthermore, the increased responses to the polypeptides were not observed after the administration of the α-adrenoceptor blocking agent, phentolamine. This indicates that the increased responses are a specific property of phenoxybenzamine and not related to α-receptor blockade.

Finally the possible physiological significance of the actions of the three polypeptides on splenic smooth muscle must be considered. There is clear evidence in the cat that both angiotensin and vasopressin contribute to the splenic responses in haemorrhage (Greenway & Stark, 1969; Cohen, Sitar, McNeill & Greenway, 1970; Stark, McNeill & Greenway, 1971). The contribution of both polypeptides appears to be restricted to the vascular response. There is no direct evidence in the dog but studies in this species on the release of vasopressin (Schrier, Verroust, Jones, Fabian, Lee & de Wardener, 1968; Rocha e Silva & Rosenberg, 1969; Biro, Forsling & Wilmott, 1972) and angiotensin (Regoli & Vane, 1966; Hodge, Lowe & Vane, 1966) in response to haemorrhage suggest that the arterial concentrations may be sufficient to increase splenic vascular resistance (Davies *et al.*, 1968a). The increased plasma concentrations would have little action of the capsular smooth muscle since this would be almost maximally contracted by the concomitant increase in both direct sympathetic activity and circulating catecholamine levels.

This work was supported by a grant from the Medical Research Council.

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THE EFFECTS OF ADENOSINE TRIPHOSPHATE AND ADENOSINE DIPHOSPHATE ON TRANSMISSION AT THE RAT AND FROG NEUROMUSCULAR JUNCTIONS

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1 The effects of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) were investigated on evoked end-plate potentials (e.p.ps) and on miniature end-plate potentials (min. e.p.ps) recorded from muscle fibres of the rat diaphragm and the frog sartorius.

2 ATP and ADP decreased the quantum content of the e.p.ps and the frequency of the min. e.p.ps. The maximum effects produced by the two substances were similar.

3 The potency of ATP was found to be similar to that of adenosine. In the presence of adenosine, in a concentration producing its maximum effect, the addition of ATP had no further effect. This is compatible with the idea that ATP acts in the same way as adenosine.

Introduction

It has been shown that adenosine and adenosine monophosphate (AMP) decrease the amount of transmitter released from nerve endings at the neuromuscular junction in the rat diaphragm (Ginsborg & Hirst, 1972; Ginsborg, Hirst, Maizels & Walker, 1973). At many sites where these substances act it is found that adenosine triphosphate (ATP) and adenosine diphosphate (ADP) are also effective, and frequently more potent than adenosine (see e.g. Burnstock, 1972). It therefore seemed of interest to compare the effects of ATP and ADP with those of adenosine. A brief account of some of the results has already appeared (Ribeiro & Walker, 1973).

Methods

The experiments were carried out, at room temperature, on isolated preparations of the rat phrenic nerve-diaphragm or of the frog nerve-sartorius. The rats used were Piebald Viral Glaxo or Wistar strains, of either sex and of not more than 200 g in weight; the frogs were *Rana temporaria*. The preparations were mounted in a Perspex chamber through which the solutions flowed continuously at a rate of 2 to 3 ml/min via a roller pump (Watson Marlow); the bath volume was 3 ml, the level being kept constant by suction. Solutions were changed by transferring the inlet tube of the pump from one flask to another.

End-plate potentials (e.p.ps) were recorded in the conventional way (Fatt & Katz, 1951) with intracellular electrodes filled with 3M KCl and of 10 to 20 MΩ resistance; the bath electrode was of the kind described by Beranek, Martin & Wickelgren (1970). Evoked responses to 64 or 128 stimuli were averaged, after amplification, with a Biomac 1000 computer. The output of the computer was coupled to a pen recorder. The nerve was stimulated at a constant rate throughout the experiments and at least 30 min to one hour before recording was begun. Miniature end-plate potentials (min. e.p.ps) were recorded on continuously moving film throughout the period required for obtaining an average evoked response. Quantum contents were estimated as the ratio of the average amplitude of the evoked e.p.ps to the average amplitude of the min. e.p.ps recorded during the same period. Where necessary in the calculation of quantum contents, correction was made for 'non-linear summation' by applying Martin's (1955) formula $m = E\bar{U}/\bar{u}(E - \bar{U})$, where $\bar{U}$ is the average amplitude of the evoked e.p.p., $\bar{u}$ is the mean amplitude of the min. e.p.ps and E is the driving force for the action of the transmitter. E was assumed to be 55 mV in the rat and 75 mV in the frog (see Hubbard, Llinás & Quastel, 1969; Ginsborg & Hirst, 1972).

The usual procedure was to continue to record averages in the same solution until a stable value was obtained, i.e. until two successive averages differed by less than 5%.

The bathing solution for the rat diaphragm contained (mM) NaCl 117, KCl 5, $NaHCO_3$ 25,

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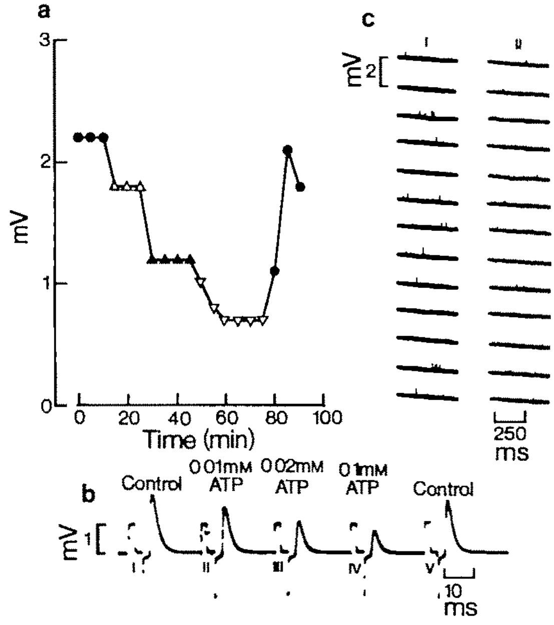

Figure 1 Effect of ATP on the average amplitude of end-plate potentials (e.p.ps) and the frequency of miniature end-plate potentials (min. e.p.ps) (rat diaphragm). Solutions contained 3 mM Ca^{2+} and 30 mM Mg^{2+} which prevented muscle action potentials and twitches. (a) The ordinates are the computed averages of 128 successive e.p.ps at 0.5 Hz and the abscissae are the times the averaging began. Control (●); ATP 0.01 mM (△); 0.02 mM (▲); 0.1 mM (▽). (b) Pen-recorder traces of averages corresponding to (i) 5 (ii) 20 (iii) 40 (iv) 70 (v) 85 min for time in (a). Each response is preceded by a 1 mV calibration pulse. (c) Samples of min. e.p.ps: they correspond to (i) 5 and (ii) 70 minutes.

NaH_2PO_4 1.2, glucose 11, and the concentrations of Ca^{2+} and Mg^{2+} were adjusted to prevent twitches of the muscle in response to nerve stimulation. The bathing solution for the frog sartorius contained (mM) NaCl 117, KCl 2.5, NaH_2PO_4 1 and Na_2HPO_4 1. The concentrations of Ca^{2+} and Mg^{2+} were adjusted as for the rat diaphragm. In a few experiments the muscle twitches were prevented by addition of tubocurarine to a solution containing 1.8 mM $CaCl_2$ and 1 mM $MgCl_2$. The drugs used were ATP, ADP, adenosine (Sigma) and tubocurarine (Burroughs Wellcome).

Results

The effects of ATP on transmitter release

Figure 1 illustrates the effect of ATP in concentrations of 0.01, 0.02 and 0.1 mM on the amplitude of the e.p.ps recorded from a muscle fibre of the rat diaphragm. The e.p.ps (Figure 1b) were evoked by stimulation of the phrenic nerve at the rate of 0.5 Hz. Groups of 128 successive e.p.ps were averaged automatically and the averages recorded at intervals of about 5 minutes. The min. e.p.ps (Figure 1c) were photographed during the averaging period (i) before, and (ii) at about 70 min after the perfusion solution had been changed to one containing ATP. As can be seen, ATP reduced the amplitude of the e.p.ps and the frequency of the min. e.p.ps. The average amplitude of the min. e.p.ps was not affected, indicating that ATP had no post-junctional effect. The reduction in the e.p.p. amplitude must thus be attributed to a reduction in quantum content, i.e. to a reduction in the amount of transmitter released.

Similar experiments were carried out on 15 fibres and the results are summarized in Table 1. It can be seen that 0.2 mM causes no greater reduction in quantum content than 0.1 mM ATP. There thus appears to be a maximum effect (corresponding to an approximately 50% reduction). This was confirmed in a subsequent experiment (see Figure 2) in which 4 mM had no greater effect than 0.2 mM ATP. From the experiments where several concentrations were tested on the same fibre, it appeared that the maximum effect requires between 0.02 and 0.1 mM ATP, although, exceptionally, it was found that in a few fibres 0.01 mM was sufficient. The reduction in the frequency of min. e.p.ps appears also to be concentration dependent.

No tachyphylaxis to the action of ATP was observed. Thus no diminution was seen in its effect on the e.p.p. during a period of 3 h perfusion by 0.2 mM ATP (cf. Mihich, Clarke & Philips, 1964).

Table 2 summarizes the results from 16 end-plates of the frog sartorius preparation, exposed to ATP in the same concentrations as used for the rat diaphragm. At all the end-plates, ATP reduced the amplitude of the e.p.ps. Min. e.p.ps were not recorded in the majority of experiments, but in those in which they were recorded, their mean amplitude was unchanged by ATP. It was therefore assumed that the amplitudes of the e.p.ps (Table 2, col. 2b) were directly proportional to the quantum contents. There were too few results for a detailed analysis, but it appeared that, as in the rat, the lower concentrations of ATP, i.e. 0.01 and 0.02 mM have submaximum effects on the quantum content.

The results so far described suggest that the percentage reduction caused by ATP of the quantum content of the e.p.p. is independent of its initial level up to a quantum content of about

Table 1 Effect of ATP on the quantum content of the end-plate potential (e.p.p.) and on the frequency of miniature end-plate potentials (min. e.p.ps) recorded from rat diaphragm fibres

Concentration of ATP (mM)	*Reduced quantum content as fraction of control value* *Mean, range, (number of expts)*	*Reduced frequency of min. e.p.ps as fraction of control value* *Mean, range, (number of expts)*
0.01	0.69 0.46 to 0.80 (7)	0.80 0.66 to 0.96 (5)
0.02	0.63 0.51 to 0.77 (5)	0.78 0.59 to 1.0 (5)
0.1	0.48 0.35 to 0.63 (9)	0.71 0.64 to 0.78 (2)
0.2	0.50 0.44 to 0.60 (7)	0.57 0.31 to 0.73 (7)

Neuromuscular transmission was blocked by bathing the preparation in solutions containing either low calcium or high magnesium concentrations. The ranges were from 0.2 mM Ca^{2+}, zero Mg^{2+} to 4 mM Ca^{2+}, 30 mM Mg^{2+}. The mean amplitude of the e.p.ps varied from about 0.3 to 4 mV with corresponding mean quantum contents from 0.5 to 20. In some of the experiments where the quantum content was low it was estimated by counting 'failures' (see del Castillo & Katz, 1954). For any one ATP concentration no obvious differences were seen between the results with the different concentrations of Ca or Mg or with different initial quantum contents.

Table 2 Effect of ATP on the quantum content of the end-plate potential (e.p.p.) and on the frequency of miniature end-plate potentials (min. e.p.ps) recorded from frog sartorius fibres

(1) *Concentration of ATP (mM)*	(2) *Reduced quantum content as fraction of control value* *Mean, range, (number of expts)* *a*	 *b*	(3) *Reduced frequency of min. e.p.ps as fraction of control value* *Mean, range, (number of expts)*
0.01	0.68 0.61 to 0.74 (2)	0.71 0.59 to 0.88 (8)	0.79 0.69 to 0.87 (3)
0.02	0.60 0.48 to 0.72 (2)	0.71 0.53 to 0.81 (5)	0.71 0.69 to 0.73 (2)
0.1	— —	0.50 0.40 to 0.60 (2)	— —
0.2	0.50 0.38 to 0.62 (8)	0.51 0.35 to 0.76 (21)	0.71 0.62 to 0.83 (3)

Neuromuscular transmission was blocked by bathing the preparation in solutions containing either low calcium or high magnesium concentrations. The ranges were from 0.2 mM Ca^{2+}, zero Mg^{2+} to 4 mM Ca^{2+}, 32 mM Mg^{2+}. The mean amplitude of the e.p.ps varied from about 0.7 to 4.8 mV with mean quantum contents from 1.4 to 15.3. The values in column 2a were obtained by direct measurement or by counting 'failures'. In column 2b the values were obtained from the amplitudes of the e.p.ps.

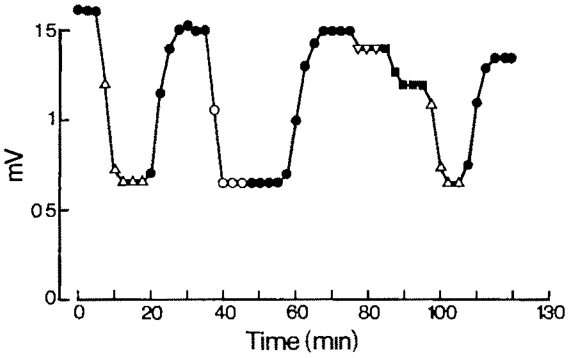

Figure 2 Effect of ATP in the presence of tubocurarine (rat diaphragm). The ordinates are the averages of 128 successive e.p.ps at the times shown. Solutions contained 2.5 mM Ca^{2+}, 1.2 mM Mg^{2+} and 2×10^{-6}M tubocurarine. Control (●), ATP 0.2 mM (△), 4.0 mM (○), 0.05 mM (■), 0.02 mM (▽).

20 (see Table 1). It was of interest to see if ATP was equally effective at higher quantum contents. Figure 2 shows that this was the case. In two experiments where tubocurarine (2×10^{-6}M in the rat and 2×10^{-5}M in the frog) was present, in solutions containing normal concentrations of calcium and magnesium, reductions to about 0.4 of the control value were produced by ATP. These results are similar to those obtained with the lower quantum contents.

Comparison between the effects of ADP, ATP and adenosine

The effects of ADP were investigated at 4 end-plates of the rat diaphragm and 5 end-plates of the frog sartorius. The concentrations used were the same as those of ATP and the results obtained were similar. It is already known that the magnitude of the effect of adenosine (Ginsborg & Hirst, 1972) is also similar to that of ATP and it seemed of interest to make a closer comparison of the maximum effects produced by the three substances. In view of the large scatter in individual results (see e.g. Tables 1 and 2), ATP and ADP were each compared directly with adenosine. From Figures 3 and 4 it can be seen that the three substances produce identical effects.

Comparison of the potencies of ATP and adenosine

The potencies of ATP and adenosine were compared by finding the concentrations necessary to produce approximately equal submaximum effects on e.p.ps recorded from the same endplate. In an experiment on the rat diaphragm bathed in a solution containing 3 mM Ca^{2+} and 30 mM Mg^{2+} it was found that 0.005 mM adenosine caused a smaller reduction than 0.01 mM ATP and that 0.005 mM ATP caused a smaller reduction than 0.01 mM adenosine. Hence adenosine must be less than twice as potent as ATP and ATP must be less than twice as potent as adenosine. Thus ATP and adenosine do not differ in potency by more than a factor of 2.

The effect of ATP in the presence of adenosine

The similarities in the potencies and in the maximum effects of ATP, ADP and adenosine strongly suggest that these substances all act on the nerve terminal in the same way. Further evidence which supports this idea is shown in Figure 5. In the experiment illustrated, adenosine in a concentration producing its maximum effect was first applied. The addition of ATP in a similar concentration now produced no further effect. This is consistent with the idea that the two substances act by a common saturable mechanism.

Discussion

The effect on transmitter release from motor-nerve terminals of a number of nucleotides and nucleosides has now been tested. Of these adenine, inosine, guanosine, uridine and cytidine (cytidine, not cystine as misprinted in the original communication) (Ginsborg *et al.*, 1973) were inactive; adenosine (Ginsborg & Hirst, 1972), AMP (Ginsborg *et al.*, 1973) ATP and ADP (present results) were active to the same extent and in the same concentrations. The same specificity obtains for substances which increase the 3′,5′-cyclic AMP content of cerebral cortex slices (Sattin & Rall, 1970). This is of interest in view of the idea frequently put forward that acetylcholine output requires the presence of cyclic AMP in the nerve terminals (e.g. Rasmussen & Tenenhouse, 1968). Whether there is any connection between transmitter release and cyclic AMP is an open question. Thus the similar specificities might only be coincidental; on the other hand a common receptor might mediate both an increase in cyclic AMP and whatever process is ultimately involved in reduction of transmitter release (Ginsborg & Hirst, 1972). There seems at present however no reason to believe that cyclic AMP is necessarily involved in the process of transmitter release (see also Miyamoto & Breckenbridge, 1974).

The present results also show that the receptors associated with the effect of adenosine and its derivatives on transmitter release from motor

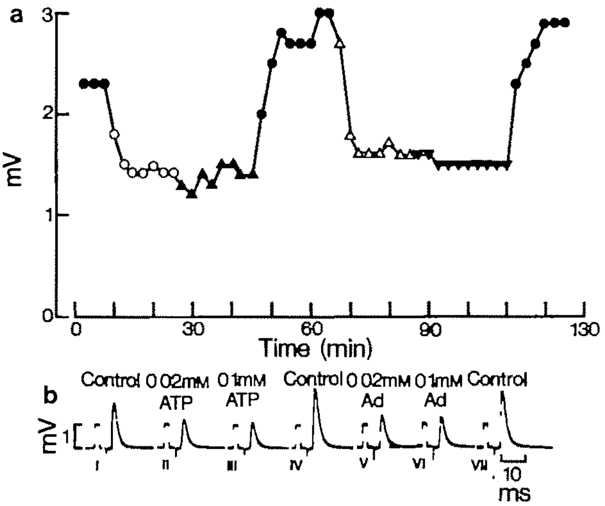

Figure 3 Comparison of effects of ATP with adenosine (Ad), (rat diaphragm). In (a) the ordinates are the averages of 64 successive e.p.ps. Control (●); ATP 0.02 mM (○), 0.1 mM (▲); adenosine 0.02 mM (△), 0.1 mM (▼). (b) Pen-recorder traces corresponding to 5, 17.5, 30, 52.5, 77.5, 97.5 and 122.5 min respectively (i-vii) in (a). For details see Figure 1.

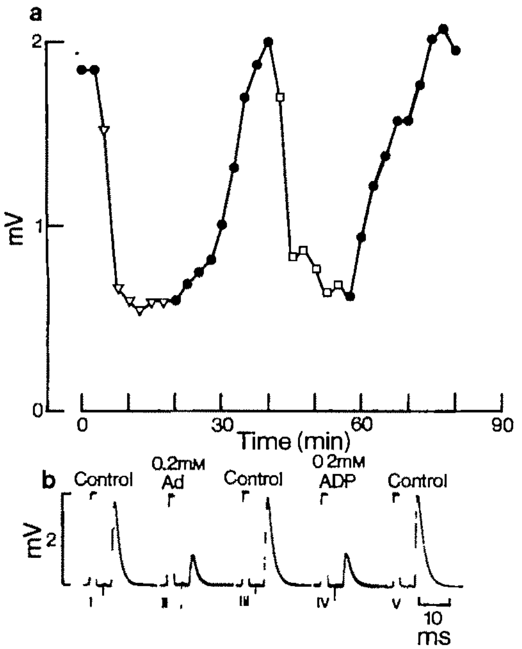

Figure 4 Comparison of effects of ADP with adenosine (Ad), (rat diaphragm). In (a) the ordinates are the averages of 64 successive e.p.ps. Control (●); adenosine 0.2 mM (▽); ATP 0.2 mM (□). (b) Pen-recorder traces corresponding (i-v) to 2.5, 12.5, 37.5, 52.5 and 75 min respectively in (a). For details see Figure 1.

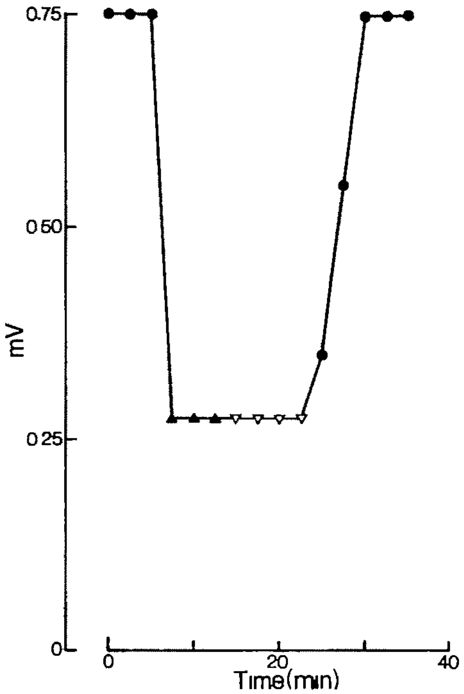

Figure 5 Effect of ATP in the presence of adenosine. Each substance was used in a concentration that alone produced a maximum effect (rat diaphragm). Ordinates are the averages of 128 successive e.p.ps. Solutions contained 2 mM Ca^{2+} and 18 mM Mg^{2+}. Control (●); adenosine 0.2 mM (▲); adenosine 0.2 mM plus ATP 0.2 mM (▽).

nerve are different from the smooth muscle receptors. For example, as inhibitors of spontaneous activity of taenia coli (Axelsson & Holmberg, 1969) ATP and ADP are 3 to 4 times more potent than adenosine and as inhibitors of coronary vessels (Winbury, Papierski, Hemmer & Hambourger, 1953; Wolf & Berne, 1956) they are about 100 times more potent than adenosine. As inhibitors of potassium induced contractures of taenia coli (Axelsson & Holmberg, 1969) adenosine, ATP and ADP are, as in the present experiments, equipotent; but in that situation adenine is also a potent inhibitor whereas it has no effect on transmitter release from the motor nerve.

We should like to thank Dr B.L. Ginsborg for helpful suggestions and discussion. One of us (J.A.R.) wishes to thank Professor E.W. Horton for his hospitality. J.A.R. is a Gulbenkian Foundation Fellow.

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(Received December 3, 1974.
Revised January 21, 1975)

PROCEEDINGS OF THE British Pharmacological Society

26th-27th March, 1975

WELSH NATIONAL SCHOOL OF MEDICINE CARDIFF

COMMUNICATIONS

In communications with more than one author, an asterisk (*) denotes the one who presented the work.

A possible mode of action of piperazine

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Histamine, a putative neuromuscular transmitter substance in nematodes, has been identified in the tissues of *Ascaris suum* (Miyagawi, 1961; Phillips, Sturman & West, 1975). In the following study, the effect of the anthelmintic drug, piperazine, on the histamine content of whole, mature, female worms has been determined using both fluorimetric and biological techniques.

The mean (± s.e. mean) histamine content of 20 control Ascaris (obtained from the intestines of freshly slaughtered pigs) after incubation for 24 h at 38°C in modified Baldwin Ringer solution (Baldwin & Moyle, 1947) was 104 ± 9 ng/g. When concentrations of piperazine (from 0.05% to 0.2%) were included in the incubation mixture, reductions of up to 16% in the histamine content were found. Partial paralysis of the parasites occurred at the highest concentration. When the concentration of piperazine was increased to 0.5%, the histamine content was raised slightly above the control value and total immobilization was noted.

The histamine content of *Ascaris suum* incubated in Ringer solution containing 10 μg/ml histamine increased to 368 ± 49 ng/g and it increased further to 560 ± 32 ng/g (a significant increase of 52%) when 0.05% piperazine was present. In the presence of 0.1% piperazine and histamine, the histamine content was raised over two and a half times although this value was not increased further when 0.2% piperazine was included in the incubation mixture. Paralysis of the parasites was always greater when histamine was present with the piperazine.

These results indicate that piperazine tends to lower the histamine content of *Ascaris suum* and in high concentrations it immobilizes the parasites. Low concentrations of piperazine, however, become more effective in producing muscular relaxation when histamine is included in the incubation mixture, and expulsion of the parasites from the host's intestine would then be assisted. As the histamine content of pig intestinal fluid is 3-10 μg/ml, the experimental conditions used reflect the normal environmental conditions of *Ascaris suum* as regards the histamine levels. The mechanism of action by which the histamine content of the worm is increased by piperazine in the presence of histamine remains uncertain but increased absorption across the body wall and relaxation of the muscle around the mouth (thereby increasing ingestion) are possibilities.

We would like to thank the Dunmow Flitch Bacon Factory for providing facilities for collecting *Ascaris suum* samples. This work was carried out during the tenure of an A.R.C. grant (J.L.P.).

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Site of action of an anti-inflammatory fraction from normal human plasma

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A fraction isolated from normal human plasma contains a substance of low molecular weight, below 500, which shows anti-inflammatory activity in animal tests in which the emigration of circulating leucocytes is a major factor (Ford-Hutchinson, Smith, Elliott, Bolam, Walker, Lobo, Badcock, Colledge & Billimoria, 1975). We have now studied its effects on the release of complement-derived chemotactic factors and anaphylatoxin from rat serum.

Preparations of the plasma fraction which caused a significant reduction in the accumulation of leucocytes in sponges implanted subdermally in the intact rat were tested at the same time for their effects on the directed migration of isolated rat leucocytes using the Boyden chamber technique (Goetzl & Austen, 1972) and on the production of anaphylatoxin by the method of Kleine, Poppe & Vogt (1970). Antigen-antibody complex was used as the activator of the classical pathway of complement and zymosan and *E. coli* endotoxin as activators of the alternate pathway.

The results (Table 1) show that the plasma fraction inhibited the release of chemotactic factors and anaphylatoxin when complement was activated by zymosan or endotoxin but not by antigen-antibody. However, there was no interference with the actions of the released chemotactic factors and anaphylatoxin on either the rat peripheral leucocytes or the isolated guinea-pig ileum. It is suggested that the active substance in the plasma fraction inhibits the C3 activator system in rat serum (Götze & Müller-Eberhard, 1971). This effect may be of some significance because of the recent observation (Goldstein & Weissmann, 1974) that leucocyte lysosomes contain a material causing a non-immune activation of the complement system thus amplifying the inflammatory response through a positive feed-back loop mechanism.

This work was supported by grants from the National Research Development Corporation, the King's College Hospital and Medical School Research Committee, the Wates Foundation, and the Nuffield Foundation.

Table 1 Effects of plasma fraction on the release of chemotactic and anaphylatoxic activities from rat and guinea-pig serum.

Addition to serum	*Chemotaxis (no. of cells per high power field)*	*Anaphylatoxin (% change: activator = 100)*
Zymosan	14.7 ± 1.7 (9)	
Zymosan + PF	8.6 ± 0.6 (9)*	−40 ± 8 (5)*
Endotoxin	22.4 ± 0.7 (9)	
Endotoxin + PF	13.2 ± 0.7 (9)*	
Antigen-antibody	14.9 ± 1.1 (9)	
Antigen-antibody + PF	15.1 ± 0.4 (8)	+2 ± 3 (3)

Results given as mean ± s.d., number of separate experiments in brackets. PF, plasma fraction (0.1 ml), * $P < 0.001$, significant difference from the corresponding value with activator alone.

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Time-dependent potentiation and inhibition by 5-hydroxytryptamine of platelet aggregation induced by ADP

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Platelet aggregation by adenosine diphosphate (ADP) can be either potentiated or, under certain conditions, inhibited by 5-hydroxytryptamine (5-HT) (Baumgartner & Born, 1968). We have investigated these opposing effects. Platelet aggregation was measured in human citrated platelet-rich plasma by the photometric method (Born, 1962). The uptake of 5-HT into platelets was measured with 5-HT labelled with ^{14}C.

In the presence of 5-HT, platelet aggregation by ADP was first accelerated and then decelerated (Figure 1). The acceleration was greatest when 5-HT and ADP were added simultaneously. With increasing intervals between the addition of 5-HT and the subsequent addition of ADP, the potentiation disappeared and was followed by inhibition. The inhibition increased for 10 min and then decreased; after 30 min the aggregation velocity was again similar to that of controls.

Earlier work (Born, Juengjaroen & Michal, 1972) showed that platelet aggregation by 5-HT is inhibited strongly by methysergide ($K_i \approx 0.03\ \mu M$) and weakly by imipramine ($K_i \approx 10\ \mu M$) whereas the uptake of 5-HT by platelets is inhibited strongly by imipramine ($K_i \approx 0.3\ \mu M$) and weakly by methysergide ($K_i \approx 125\ \mu M$). Methysergide at 0.25 μM completely inhibited both potentiation and inhibition by 5-HT of aggregation by ADP. Imipramine at 0.5 μM, and also at 5 μM which inhibited 5-HT uptake completely, only partially prevented the potentiation but did not prevent the inhibition nor the subsequent recovery. Ouabain at up to 100 μM influenced neither potentiation nor inhibition.

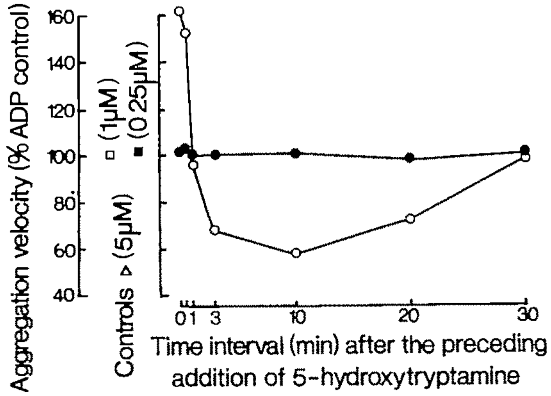

Figure 1 Aggregation velocity of human platelets (expressed as percentage of ADP control) produced by 1 μM ADP at different intervals after preceding addition of 5 μM 5-HT in the presence (●) and the absence (○) of 0.25 μM methysergide (MeS). For comparison aggregation velocities of 5-HT (△) and ADP alone (□) and ADP in the presence of methysergide (■) are indicated on the left of the figure. Methysergide was added 5 min before 5-HT or ADP; all samples were incubated for 30 min at 37° C before the addition of the aggregating agent.

Further evidence that both potentiation and inhibition of aggregation velocity by 5-HT are independent of the uptake of 5-HT was obtained with 5-methoxy-α-methyltryptamine (5 μM) which is not taken up by platelets (Born *et al.*, 1972) yet produced both potentiation and inhibition just like 5-HT itself. Therefore these effects of 5-HT are unrelated to its uptake by platelets but rather to a reversible effect of 5-HT on the plasma membrane.

M. Motamed is in receipt of a fellowship from the Wellcome Trust. F. Michal is a Senior Research Fellow of the British Heart Foundation.

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The effect of histamine on tissue blood flow in the cat

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Histamine lowers blood pressure in the cat by causing vasodilatation. This response involves histamine H_1- and H_2-receptors (Owen & Parsons, 1974; Flynn & Owen, 1974). The experiments described in this communication were designed to help our understanding of the distribution of histamine receptors through the peripheral circulation.

Experiments have been made in cats, body weight 1.2-2 kg, anaesthetized by an intraperitoneal injection of chloralose 40 mg/kg and urethane 600 mg/kg. The trachea was cannulated. Blood pressure was measured from the right brachial artery. Histamine was infused via the right brachial vein. Heart rate was measured from the blood pressure pulse. Cardiac output and tissue blood flows were measured using radioactive microspheres, 25 μ diameter (3M Company). Detailed accounts of the microsphere technique and validation of its principles have been described elsewhere (Rudolph & Heymann, 1967; Neutze, Wyler & Rudolph, 1968; Wagner, Rhodes, Sasaki & Ryan, 1969; Warren & Ledingham, 1974).

Two injections of microspheres, about 100,000 spheres per injection, were made in each experiment. During a control period microspheres labelled with ^{46}Sc were injected. Infusions of histamine (10, 33 or 100 nmol kg^{-1} min^{-1}) or saline (control experiments) were then started. The second injection of microspheres, labelled with ^{85}Sr, was made during the continued infusion, 30 min after the start of the infusion.

Histamine caused dose-dependent falls in blood pressure, but did not change heart rate or cardiac

Table 1 Effect of histamine on blood pressure and tissue blood flow

Histamine infusion nmol kg^{-1} min^{-1}	0 (saline infusions)	10	33	100
	n = 8	n = 6	n = 6	n = 6
Mean blood pressure mm Hg				
Before infusion	119.3 ± 6.5	132.3 ± 8.6	135.3 ± 8.4	113.3 ± 5.8
During infusion	108.8 ± 5.9	120.3 ± 8.4	109.8 ± 3.5	66.5 ± 2.7
	Blood flow ml min^{-1} 100 g^{-1} ± s.e.m.			
Brain	63.1 ± 6.0	72.2 ± 5.7	66.5 ± 10.2	40.5 ± 4.6
Heart	281.3 ± 32.2	361.6 ± 43.1	593.7 ± 61.5	655.6 ± 53.4
Kidneys	346.8 ± 32.5	316.2 ± 36.1	409.2 ± 37.6	238.5 ± 23.5
Stomach	17.3 ± 2.2	22.7 ± 3.2	40.1 ± 6.4	94.1 ± 18.6
Small intestine	34.8 ± 4.3	35.3 ± 3.0	44.5 ± 4.9	36.9 ± 10.2
Large intestine	50.6 ± 8.9	57.9 ± 5.6	64.1 ± 14.5	53.7 ± 11.4
*Liver	97.1 ± 8.2	109.8 ± 14.4	103.8 ± 15.2	54.2 ± 4.2
Adrenal glands	469.4 ± 81.8	444.1 ± 66.7	546.9 ± 267.8	379.5 ± 56.0
Skeletal muscle	4.4 ± 0.8	3.9 ± 0.9	3.7 ± 1.0	2.8 ± 0.3
Skin	3.5 ± 0.7	4.8 ± 0.7	3.1 ± 0.6	1.4 ± 0.2

All values from the second injection of microspheres during infusions.

The pre-infusion blood flows in all groups were not significantly different from the values shown during the infusions of saline.

* Excludes portal blood flow.

output. The changes in blood pressure and tissue blood flow caused by histamine are shown in Table 1. Blood flows to the heart and stomach were increased despite the falls in blood pressure. Blood flows to the brain, kidney, skin and liver (arterial flow only) were maintained at the lower histamine infusion rates but declined at the highest infusion rate. In the other organs blood flow was maintained despite the falls in blood pressure.

These experiments suggest that histamine does not dilate resistance vessels equally in all tissues indicating unequal distribution of histamine or histamine receptors. Although the changes in tissue blood flow during histamine infusions were due to the local vascular effects of histamine they may have been complicated by reflex vascular changes caused by the accompanying hypotension. It is hoped that these and further experiments using histamine antagonists will reveal the distribution of H_1- and H_2-receptors in resistance vessels.

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A procedure to demonstrate the release of a vasoactive metabolite by catecholamines from perfused guinea-pig hearts

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The action of catecholamines on the coronary circulation is complex. They both constrict and dilate the coronary arterioles by directly stimulating α- and β-adrenoceptors (Parratt, 1968). The accompanying positive inotropic and chronotropic responses also indirectly constrict through extravascular compression and cause a coronary vasodilatation which is attributed to the release by the increased myocardial activity, of a vasoactive metabolite (Berne, 1964). This component is the predominant and most controversial effect. Proposed transmitters of this response have included potassium ions, lactate, kinins, adenosine (Berne, 1964) and prostaglandins (Talesnik & Sunahara, 1973), with a local anoxia possibly serving as an intermediate stimulant. However, most of the evidence is circumstantial, based upon the modification of the response by other drugs. This study was undertaken to develop a procedure and apparatus that could clearly demonstrate the presence of a metabolite during the coronary vascular response to catecholamines.

Guinea-pig (350-600 g) isolated hearts were set up as described previously (Broadley, 1970) perfused at a constant rate (5 ml/min) with Krebs-bicarbonate solution (37°C) gassed with carbogen. Records of coronary perfusion pressure and isometric force and rate of contraction were obtained on a Devices M19 polygraph. The pulmonary vein and venae cavae were tied off and after trimming the lungs free, the cut pulmonary artery was cannulated enabling the perfusate to pass to a second guinea-pig perfused heart (II) (Figure 1). This was perfused at a constant rate exactly matching that of heart I and identical parameters recorded. An open reservoir serving as an air trap was included between hearts I and II. where perfusate was regassed and fresh Ringer could be added. The volume of reservoir and tubing was minimal to keep small amounts of any metabolite concentrated.

With two hearts thus connected in series, adrenaline (0.25 μg) added to heart II increased the force and rate of contraction and produced a characteristic coronary vascular response consisting of a biphasic constriction followed by the predominant vasodilatation. Adrenaline (0.25 μg) added to heart I also produced these responses,

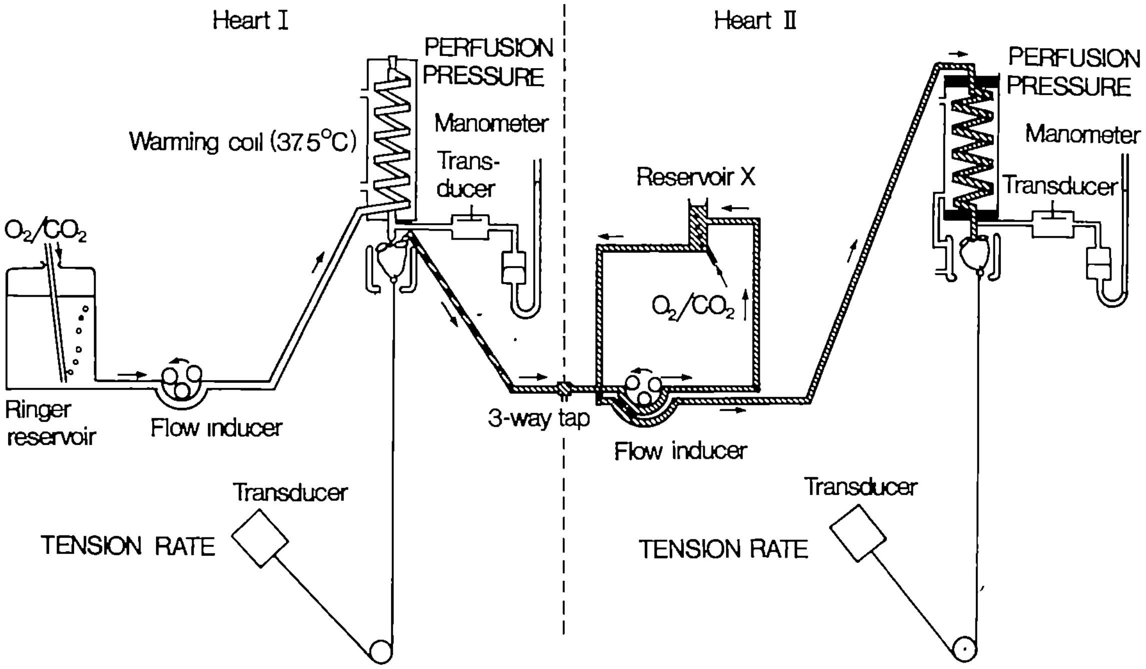

Figure 1 Diagram of apparatus for perfusing guinea-pig isolated hearts (I) and collecting the perfusate for supplying a second perfused heart (II).

but it was carried in the perfusate to heart II where it again produced similar responses, although the coronary response was sufficient to mask any effects due to a metabolite from heart I. These direct β-adrenergic effects on heart II were antagonized by propranolol (15 μg). Adrenaline repeated in heart I gave normal responses but a coronary vasodilatation followed in heart II unaccompanied by rate or force changes. This vasodilatation was abolished when the rate and force changes in heart I were also blocked by propranolol. It can therefore be attributed to a vasodilator substance released from heart I by the increased activity and not by the adrenaline itself.

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The effects of glucagon and pentagastrin on capillary filtration coefficient in the innervated jejunum of the anaesthetized cat

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Capillary filtration coefficient (CFC) measurements in the innervated cat jejunum have been shown (Richardson, 1974) to be affected by doses of some drugs which are too low to produce any appreciable effects on other variables; the present report describes the effects of two gastrointestinal hormones on these preparations. Eleven cats (mean weight 3.1 kg, range 2.7-4.0 kg) were anaesthetized with chloralose (70 mg/kg i.v.) after halothane induction, and CFC measured in loops of innervated jejunum (60 ± 14 g: mean ± s.d.) using a modification (Richardson, 1974) of the technique of Folkow, Lundgren & Wallentin (1963). All values are expressed as means ± s.e. means, and the significance of differences between paired data tested by Student's *t*-test. The control values were: for systemic arterial mean pressure 121 ± 9 mm Hg (1 mm Hg = 133 Pa), for heart rate 172 ± 12 beats/min, for jejunal blood flow 31 ± 4 ml min^{-1} 100 g^{-1}, for jejunal vascular resistance 4.58 ± 0.59 PRU (mm Hg ml^{-1} min 100 g) and for CFC 0.028 ± 0.005 ml min^{-1} mm Hg^{-1} 100 g^{-1}, values similar to those previously reported for such preparations (Richardson, 1974).

Glucagon hydrochloride (Lilly) was infused i.v. once to each of seven preparations in a dose of 0.25 μg kg^{-1} min^{-1}, producing a significant ($P < 0.01$) reduction in CFC of 55 ± 14% from 0.023 ± 0.004 to 0.009 ± 0.003 ml min^{-1} mm Hg^{-1} 100 g^{-1}. The systemic arterial mean pressure fell by 5 ± 1% ($P > 0.05$), the heart rate rose by 8 ± 6% ($P > 0.05$), the jejunal blood flow rose by 7 ± 4% ($P > 0.05$) and the jejunal vascular resistance fell by 8 ± 3% ($P > 0.05$).

Pentagastrin (Peptavlon, ICI) was infused i.v. in a dose similar to the human gastric secretagogue dose (Wormsley, Mahoney & Ng, 1966), 0.1 μg kg^{-1} min^{-1}, on one occasion to each of five preparations. The CFC was reduced by 46 ± 4% from 0.037 ± 0.008 to 0.021 ± 0.008 ml min^{-1} mm Hg^{-1} 100 g^{-1} ($P < 0.01$), whilst the systemic arterial mean pressure fell by 4 ± 3% ($P > 0.05$), the heart rate rose by 1 ± 3% ($P > 0.05$) and the jejunal blood flow fell by 4 ± 2% ($P > 0.05$) leaving the jejunal vascular resistance unchanged (0 ± 2%).

In five preparations, the glucagon infusion (0.25 μg kg^{-1} min^{-1}, i.v.) was repeated after phentolamine, 2.0 mg/kg, a dose found adequate to block the effects of exogenous α-adrenoceptor stimulants (Richardson, 1974). The effects of glucagon on systemic arterial mean pressure, heart rate, jejunal blood flow and vascular resistance did not differ from those before phentolamine ($P > 0.5$). However, glucagon now caused a rise in CFC of 38 ± 16% from 0.024 ± 0.005 to 0.031 ± 0.004 ml min^{-1} mm Hg^{-1} 100 g^{-1} ($P < 0.02$). The difference between the effects of glucagon before and after phentolamine was highly significant ($P < 0.001$).

Drug effects on CFC are due to changes either in vascular permeability or in the functional exchange vessel area brought about by alterations in the tone in the precapillary 'sphincters'. The reversal of the effects of glucagon by phentolamine points towards the conclusion that glucagon causes constriction of the precapillary 'sphincters' which is reversed to a dilator action after α-adrenoceptor blockade. Glucagon releases suprarenal catecholamines in the cat (Fasth & Hulten, 1971) and these effects may be related to this action.

This study was generously supported by the Medical Research Council.

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Cardiovascular sensitivity of experimentally diabetic and genetically obese pithed rats to autonomic agents

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The increased risk of autonomic malfunction associated with diabetes mellitus is now well established. Impaired vagal function has been demonstrated in diabetic patients by Dotevall, Fagerberg, Langer & Walan (1972) and Wheeler & Watkins (1973). Hyper-responsiveness to noradrenaline has been demonstrated in vascular tissue from experimentally diabetic animals, e.g. in perfused rat hindquarters by Brody & Dixon (1964) and in the isolated rabbit aorta by Cseuz, Wenger, Kunos & Szentivany (1973). In this communication a comparison between the differences in sensitivities of the cardiovascular systems of two week streptozotocin (60 mg/kg i.v.) and one week alloxan (50 mg/kg i.v.) diabetic CFE male rats and their untreated controls (10, 5 and 10 respectively) and between genetically obese (Zucker strain) and their non-obese litter mates (6 and 7 respectively) is presented. The animals were pithed and dose-response curves to noradrenaline, isoprenaline and acetylcholine constructed. Animals treated with the diabetogens were only considered diabetic if their blood glucose was found to be above 250 mg per 100 ml blood (estimation of blood glucose was by a microcolorimetric copper reduction method). Falls in mean blood pressure caused by 1 and 3 μg/kg acetylcholine were significantly ($P < 0.05$) reduced in both diabetic groups compared with their controls. The genetically obese rats, however, appeared to have similar sensitivities to their non-obese litter mates. Rises in mean blood pressure caused by 100 ng, 300 ng, 1 μg and 3 μg/kg noradrenaline were all significantly ($P < 0.01$) reduced in the streptozotocin but not in the alloxan diabetic group. The pressor response to noradrenaline was similar in the obese group compared to their non-obese litter mates.

Changes in blood pressure due to isoprenaline were not significantly different in any of the three test groups compared with their controls. Changes could, however, have been obscured by the rather large standard errors obtained.

The lack of increased noradrenaline sensitivity in alloxan diabetic rats contrasts with the results of Brody & Dixon (1964).

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Inhibition of the effects of weight-reducing drugs in guinea-pigs by Vitamin C

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Administration of fenfluramine produces loss of weight and anorexia in guinea-pigs (groups of 5) on a scorbutogenic diet (Odumosu & Wilson, 1974). This effect occurs in consequence of the immediate tissue depletion of ascorbic acid produced by the drug. Its anti-obesity action is inhibited by administration of supplementary Vitamin C. The change in body weight and anorexia after administration of saline, fenfluramine, diethylpropion or mazindol were investigated over a period of 72 h, in guinea-pigs, after 24-h fasting. The drugs were given i.p. in doses of fenfluramine (F) 15 mg/kg, diethylpropion (D) 50 mg/kg, mazindol (M) 15 mg/kg, with or without Vitamin C 30 mg/kg s.c. Body-weight increased by 5% after 72 h in the guinea-pigs receiving saline. In those receiving Vitamin C, weight increased by 8%, confirming the finding of Williams & Hughes (1972). Fenfluramine (15 mg/kg) caused a 5% reduction in body weight after 7 h which had decreased to 6% by 24 hours. After 60 h the guinea-pigs in the F group had regained their initial weight. Administration of Vitamin C to guinea-pigs in the F group caused them to gain weight after 7 h so that the initial weight was regained after 48 hours. Mazindol caused 2% (not significant) loss of weight which had been regained after 7 hours. After 72 h guinea-pigs in the M group were as heavy as the group receiving the Vitamin C supplement alone. Administration of Vitamin C to guinea-pigs in the M group completely inhibited weight loss.

Vitamin C had increased food intake to 140% of that in the saline treated group 2 h after its administration. Fenfluramine inhibited food intake for 5 h in the same fashion as mazindol and diethylpropion. After 48 h food intake was 85% of that in the saline treated group. Supplementary Vitamin C caused guinea-pigs in the D group to increase their food intake 1 h sooner than the F group. In the M group Vitamin C restored food intake to 60% of that in the saline group within 2 h of administration. Administration of Vitamin C can differentiate between the anti-obesity and anorectic actions of weight-reducing drugs. It inhibits the anti-obesity action, but has little effect on the anorectic action, of fenfluramine and diethylpropion. Vitamin C inhibits the anti-obesity action of mazindol and reduces its anorectic effect.

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Rat liver tryptophan pyrrolase activity in iron deficiency anaemia

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Liver tryptophan pyrrolase (TP) is one of the peripheral factors affecting 5-hydroxytryptamine (5-HT) synthesis (Badawy & Evans, 1974) and is inversely-related to brain (5-HT) concentration (Curzon, 1969). The haem activator of TP contains Fe, and we therefore examined the effect of iron deficiency on TP activity and on three of its regulatory mechanisms.

Male Wistar rats (Tucks, Rayleigh, Essex) were made mildly- or severely-Fe-deficient by the diet of McCall, Newman, O'Brien, Valberg & Witts (1962). Control rats received the same diet supplemented with Fe. The deficiency was verified by haematological tests. The determination of TP activity in the absence (holoenzyme) or the presence (total enzyme) of added haematin, and the doses of injected compounds have previously been described (Badawy & Evans, 1973). The haem-free apoenzyme was calculated by difference. The results in mildly- and severely-Fe-deficient rats, as well as in their respective controls, were similar and were therefore pooled.

Control rats ('A' groups) gave typical results

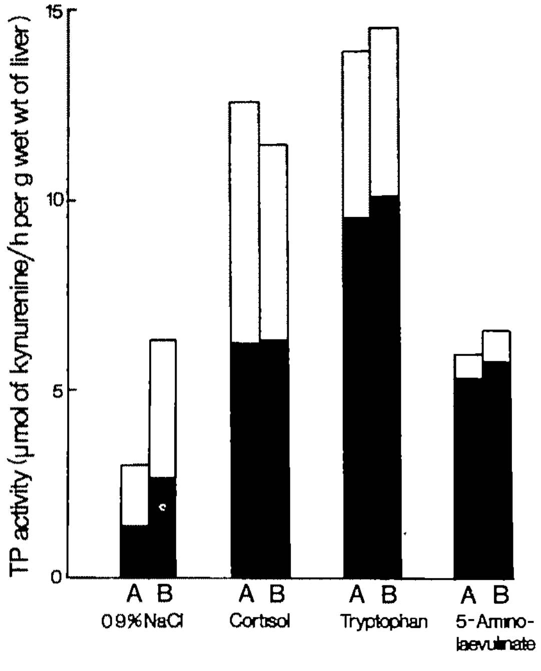

Figure 1 Comparison of tryptophan pyrrolase activities in iron-deficient and control rats.
The enzyme activity was determined at 4 h after intraperitoneal administration of various compounds. Closed columns represent holoenzyme whereas open ones apo-enzyme activity. The sum of the two columns gives the total activity. A, Control rats; B, Iron-deficient rats.

(Figure 1). The basal (0.9% NaCl) holoenzyme was less than half the total activity. The induction of TP by cortisol (and stressful agents that release corticosteroids) involves increased apo-TP synthesis (Schimke, 1969). Here, the increase in the total TP activity is matched by a proportionate rise in that of the holoenzyme. A relatively larger rise in the holoenzyme is produced by tryptophan, which acts mainly by activating pre-existing apoenzyme. 5-Aminolaevulinate, acting via haem (Badawy & Evans, 1973), causes a strong saturation of apo-TP; the majority of activity being as holoenzyme.

Fe-deficient rats lost weight. The basal holoenzyme and total enzyme activities (after 0.9% NaCl, 'B' groups in Figure 1) rose in Fe deficiency by 93-110% ($P = 0.02$-0.001). Similar rises have been reported (Badawy & Evans, 1973) in starved rats which also exhibited weight loss. The stress condition of Fe-deficient rats, and the qualitative resemblance of the rise in their TP activities to that by cortisol suggest that corticosteroids may be involved in the effect of Fe deficiency. Cortisol, tryptophan and 5-aminolaevulinate were equally effective in increasing TP activities in Fe-deficient ('B' groups) and control ('A' groups) rats. This suggests that TP induction by hormones (cortisol), and its activation by substrate (tryptophan) or cofactor (haem) are not impaired in Fe deficiency.

The present and other results (Symes, Missala & Sourkes, 1971; Callender, Grahame-Smith, Woods & Youdim, 1974; Bailey-Wood, Blayney, Muir & Jacobs, 1975) suggest that enzymes requiring haem- or non-haem-Fe may be increased, unaltered or decreased in Fe deficiency. The activities of liver TP, brain tryptophan hydroxylase and monoamine oxidase(s) may all control the level of brain 5-HT in Fe-deficient rats. Work on these aspects is planned.

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The measurement of serum morphine levels by radioimmunoassay following oral administration of diamorphine or morphine

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The development of radioimmunoassay for morphine and related substances has resulted in greater opportunities for studying such compounds (Spector & Parker, 1970; Catlin, Cleeland & Grunberg, 1973; Morris, Robinson, Piall, Aherne & Marks, 1974). We report a method currently in use at the Department of Biochemistry, University of Surrey, and present preliminary data relating to the measurement of serum morphine in patients with advanced malignant disease receiving either diamorphine or morphine by mouth.

Antiserum was raised in a goat to a conjugate of 6-succinylmorphine-bovine serum albumin. Because the antiserum cross reacts almost equally with diamorphine, monoacetylmorphine, morphine and codeine, results are expressed as 'morphine equivalents'. Tritiated dihydromorphine was used as the radioactive label in the assay which has been described elsewhere (Aherne, Piall, Robinson, Morris & Marks, 1974). There is no cross reactivity for normorphine and only some 10% for morphine-3-glucuronide. Other drugs being taken by the patients did not cross react.

Quantities as small as 300 pg/ml can be measured by this method.

Twenty patients received diamorphine hydrochloride and twenty-four morphine sulphate, administered orally every 4 h in an elixir. The dose of diamorphine hydrochloride ranged from 5-60 mg (median 10 mg) and the dose of morphine sulphate from 3.75-90 mg (median 19 mg). The two groups were comparable in terms of sex, age, primary site and survival. Although most blood samples were obtained before or shortly after the 9 a.m. drug round, five specimens were obtained more than 40 min later. At the time of venepuncture all the patients had received an opiate regularly for more than a week.

The serum concentrations showed a significant correlation with the dose administered. Linear correlation coefficients for the two groups were: oral diamorphine, $r = +0.91$ ($p < 0.001$), oral morphine, $r = +0.73$ ($p < 0.001$) and for both groups, $r = +0.79$ ($p < 0.001$). There was no apparent difference between the two groups in the serum concentrations measured at comparable dose levels.

Our results demonstrate that it is possible to measure unconjugated morphine in serum from patients who have been receiving either diamorphine or morphine by mouth regularly for a number of days. It is detectable both at the beginning and the end of the 4 h interval between administrations. The positive correlation between dose and serum concentration supports recently published data showing a similar association between the dose administered and urinary recovery (Twycross, Fry & Wills, 1974). Further experiments to determine rates of absorption and elimination for the two drugs are planned in both volunteers and patients.

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A microsomal oxidase system in rat intestinal mucosa

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Orally administered drugs may be metabolized before reaching the general circulation. For example, L-dopa is decarboxylated in the human gastric tissue (Rivera-Calimlin, Dujovne, Morgan, Bianchine & Lasagna, 1971); clorazepate is converted to N-desmethyldiazepam, also in the human stomach (Boehringer Ingelheim Ltd., personal communication); orally administered isoprenaline is conjugated in the gut wall during absorption (Dollery, Davies & Connolly, 1971) and propranolol has been suggested to be extensively metabolized during the first passage through the liver (Shand, Nuckolls & Oates, 1970).

In an earlier report we showed that chlorpromazine is destroyed in rat intestinal tissue *in vitro* and *in vivo* (Curry, D'Mello & Mould, 1971); this reaction is believed to occur in man (Sakalis, Curry, Mould & Lader, 1972). It is possible that a mixed-function oxidase system similar to that in liver may exist in the rat intestine, as Takesue & Sato (1968) have already demonstrated the existence of enzymes associated with such a system in the microsomal fraction isolated from the mucosa of another species, rabbit. In contrast to this, Chhabra, Pohl & Fouts (1974) were unable to detect one of the enzymes concerned, cytochrome P-450, in the mucosa of rat small intestine. We would like now to report on the separation and partial evaluation of a microsomal fraction from the mucosal cells of rat small intestine, containing cytochrome P-450.

The mucosa was scraped off 10 cm lengths of proximal rat small intestine and homogenized in 0.05 M phosphate buffer pH 7.4 containing 1.15%

KCl. The homogenate was centrifuged at 10,000 g for 20 min, followed by a further centrifugation of the supernatant at 140,000 g for 90 min, to obtain the microsomal pellet. The pellet was then resuspended in buffer and, following a further centrifugation at 140,000 g for 45 min, it was finally suspended in 0.1 M phosphate buffer pH 7.6. Cytochromes P-450 and b_5 were determined by the method of Omura & Sato (1964) and NADPH-cytochrome c reductase by the method of Phillips & Langdon (1962). The metabolism of foreign compounds was measured over 30 min at 37°C using the incubation conditions described by Mazel (1971).

The concentration of cytochrome P-450 was 9 times lower than that found in liver (intestine 0.046 ± 0.004 $\text{nmol.mg protein}^{-1}$; liver 0.43 ± 0.05), of cytochrome b_5 was 5 times lower (intestine 0.080 ± 0.014 $\text{nmol.mg protein}^{-1}$; liver 0.42 ± 0.08) and of NADPH-cytochrome c reductase was 2½ times lower (intestine 57.4 ± 5.1 $\text{nmol.mg protein}^{-1}\ \text{min}^{-1}$; liver 142 ± 3.10). Pretreatment with phenobarbitone (75 mg/kg daily, i.p.) over a period of 3 days lowered slightly the mean concentrations of cytochrome P-450 (0.031 ± 0.006 $\text{nmol.mg protein}^{-1}$) and NADPH-cytochrome c reductase (44.3 ± 4.1 $\text{nmol.mg protein}^{-1}\ \text{min}^{-1}$) but appeared to increase the concentration of cytochrome b_5 (0.108 ± 0.013 $\text{nmol.mg protein}^{-1}$). The 10,000 g supernatant was shown to be N-demethylate, ethylmorphine and aminopyrine.

This evidence suggests the presence of a mixed-function system in rat intestinal tissue similar to that found in liver.

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The acetylation of HC-3 by choline acetyltransferase

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The main action of HC-3 on cholinergic transmission is presumed to be an inhibition of the uptake of choline into the presynaptic nerve terminal (MacIntosh, Birks & Sastry, 1956). It has also been suggested that HC-3 might itself be incorporated into the nerve terminal where it may be acetylated by choline acetyltransferase (ChAc) and subsequently released as a false neurotransmitter (Rodriguez de Lores Arnaiz, Zieher & de Robertis, 1970; Hemsworth, 1971).

The acetylation of HC-3 by ChAc *in vitro* has been studied in a number of laboratories with conflicting results (Diamond & Milfay, 1972). The present experiments were therefore performed to investigate these differences in the acetylation of HC-3. Several different established methods for determination of ChAc activity were utilized to detect any acetylated product that might be formed by enzymatic acetylation.

The observed acetylation of 10^{-3}M HC-3 by soluble rat brain ChAc, compared with the acetylation of choline at the same concentration, varied between 0 and 27% depending on the procedure used for the isolation of the acetylated product.

When the reineckate method, first described by McCaman & Hunt (1965), was used for isolation of the radioactive acetylated products of incubation, HC-3 was shown to be acetylated at 26% the rate of acetylation of choline. When the sodium tetraphenylboron extraction method of Fonnum (1969) was used, HC-3 was shown to be acetylated 11% compared with the acetylation of choline. The ion-exchange method used by Diamond & Kennedy (1968) was also used for isolation of any acetylated reaction products, however with this method no acetylation of HC-3 was observed. A mercuric potassium iodide extraction procedure described by Glover & Green (1972) was also utilized for determination of radioactive acetylated product and the acetylation of HC-3, in comparison with choline, using this method was shown to be 21%.

These variations in the acetylation of HC-3, in comparison with the acetylation of choline, appear to arise as a result of differences in the recoveries of acetyl HC-3 and acetylcholine when different methods are used for the isolation of the acetylated radioactive products of incubation. Identification of acetyl HC-3 and acetylcholine was made by paper electrophoresis and the results verify that HC-3 can be acetylated by ChAc *in vitro*.

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Mechanism of accumulation of chlorpromazine in subfractions of rat brain

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Our previous findings have shown that subcellular fractions of rat brain prepared from a 10,000 g pellet are capable of accumulating chlorpromazine (CPZ) when incubated with ^{3}H-CPZ *in vitro* giving similar results to the ^{3}H-CPZ found after preparation of subfractions following i.p. injection *in vivo*. A time course study for the accumulation in subfractions prepared from cortex, mid-brain and hind-brain showed that an equilibrium situation was rapidly achieved which did not alter appreciably after the first few minutes of incubation. Further experiments have been performed to investigate the mechanism of this accumulation. Subcellular fractions were prepared on a discontinuous sucrose gradient by a method based on that of Marchbanks & Whittaker (1967) and incubated for 15 min in ^{3}H-CPZ which had been diluted down with different concentrations of unlabelled CPZ. Pellets were collected by rapid centrifugation and activity measured by liquid scintillation counting. Results showed that the amount of CPZ accumulated increased with increasing concentration up to the highest concentration studied of 10^{-3} M, and furthermore, preincubation of fractions in 10^{-3} M CPZ, followed by subsequent incubation in different concentrations of ^{3}H-CPZ as before, did not alter the amount accumulated. The amount of protein in the incubation did not appear to be a limiting factor. The possible pH dependence of the accumulation was investigated by altering the pH of the medium and this showed that, although the difference in the amount accumulated between the individual pH points tested (pH 5, 6, 7 & 8) was not significant, the difference between the accumulation at the highest and lowest pH was significant at the level $P < 0.001$.

To investigate the dependence of this accumulation on the lipid content of the subfraction, proteolipids were extracted from subfractions which had been incubated in ^{3}H-CPZ by a method based on that of Pasquini & Soto (1972) using n-butanol-water. Following 15 min incubation suspensions were spun down and the resultant pellets resuspended in 1.5 ml 50% sucrose and extracted for 2 h at room temperature with 16 ml n-butanol-water. Experiments showed a large uptake into the organic layer in the ratio 9.7 : 1 compared to partition of organic solvent against an aqueous solution of CPZ of 1 : 1. Extraction of proteolipids from subfractions followed by incubation with ^{3}H-CPZ gave similar results. Subfractions prepared from rats which had been injected with ^{3}H-CPZ (8 mg/kg i.p.) also showed a preferential uptake into the organic layer when partitioned against water in the ratio 12.5 : 1. Phospholipids, phosphatidyl inositol and phosphatidyl ethanolamine in n-butanol-water (Blaustein, 1967) incubated with ^{3}H-CPZ followed by partition against water also gave a similar partition of about 10 : 1 in favour of the organic layer.

These results have led us to believe that the phenomenon of accumulation of CPZ in rat brain subfractions is a solubility effect of the CPZ in the proteolipid of the membranes.

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The effect of L-tryptophan on changes in motor activity caused by parachlorophenylalanine

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L-tryptophan administration increases brain 5-hydroxytryptamine (5-HT) turnover without effect on either motor activity (Modigh, 1973) or sensitivity to electric shock (Hole & Marsden, 1975). Intravenous infusion of L-tryptophan into normal human subjects also has relatively little influence on the results of objective psychological tests though EEG changes were noted (Greenwood, Friedel, Bond, Curzon & Lader, 1974). It is possible that L-tryptophan may have more striking effects when 5-HT metabolism is deficient. This would be consistent with the postulated 5-HT defect in depression and the reported beneficial effect of tryptophan on it (Coppen, 1972). We have studied the effect of L-tryptophan on motor activity in rats previously given p-chlorophenylalanine (PCPA) at a dosage that increased activity (Fibiger & Campbell, 1971) while only partially inhibiting 5-HT synthesis.

Male Sprague-Dawley rats (180-200 g) were housed in groups of three under a 12 h light-dark cycle at 24° C. After 4 days the following drug schedule was adopted: (1) PCPA (150 mg/kg i.p.) followed by L-tryptophan (150 mg/kg i.p.) 24 h later. (2) PCPA (150 mg/kg) plus vehicle (2.5% Tween in 0.9% saline) 24 h later. (3) Saline (0.9%) plus L-tryptophan (150 mg/kg) 24 h later. (4) Saline plus vehicle 24 h later. In one experiment L-tyrosine was given instead of L-tryptophan. Motor activity was measured simultaneously in control and treated rats with Animex DSE activity meters. Activity recording was started 15 min after the last injection (at 9.45 or 11.45 h) and continued for 120 minutes. At the end of this period the animals were killed and brain tryptophan, 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) measured (Curzon, Joseph & Knott, 1972).

L-tryptophan alone caused no significant change in motor activity while PCPA alone caused a significant increase which was almost completely reversed by L-tryptophan but not by L-tyrosine. L-tryptophan caused increases in brain tryptophan (+545%), 5-HT (+50%) and 5-HIAA (+108%). PCPA altered brain tryptophan by −26%, 5-HT by −59% and 5-HIAA by −66%. L-tryptophan markedly diminished the biochemical effects of PCPA, both 5-HT and 5-HIAA returning towards control values (−19% and −10% respectively). While L-tyrosine caused a significant increase in tyrosine (+76%) there was no significant change in brain tryptophan, 5-HT and 5-HIAA. Similarly, the tryptophan, 5-HT and 5-HIAA values in rats given PCPA were not significantly different from those in rats given PCPA and L-tyrosine.

Fibiger & Campbell (1971) showed that the effects of PCPA on motor activity were reversed by 5-hydroxytryptophan (5-HTP). However, results with 5-HTP are not clearly interpretable as, unlike tryptophan, it can be decarboxylated to 5-HT in neurones other than those containing 5-HT (Fuxe, Butcher & Engel, 1971). The present finding that tryptophan reverses the effect of PCPA is stronger evidence that 5-HT neurones are involved. Furthermore, the results demonstrate an alteration of behaviour by tryptophan only in 5-HT deficient animals and therefore may be relevant to the effect of tryptophan in depression.

The M.R.C. are thanked for financial support.

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The effects of lithium, rubidium and caesium on the response of rats to tranylcypromine and α-methyl-*p*-tyrosine given separately or in combination

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Administration of 20 mg/kg tranylcypromine (Tc) to rats primed for three days with lithium chloride (LiCl) injections produces hyperactivity which is inhibited by para-chlorophenylalanine (Grahame-Smith & Green, 1974b). A similar syndrome is produced by combining L-tryptophan and Tc. This latter response is inhibited by α-methyl-*p*-tyrosine (αmpt) (Grahame-Smith & Green, 1974a). The effect of Tc on LiCl, rubidium chloride (RbCl) or caesium chloride (CsCl) loaded rats is reported here. Rats given LiCl, RbCl or CsCl in their diet (30 mmol/kg dry food) for 14 days and then injected s.c. with 15 mg/kg Tc exhibited marked hyperactivity. All facets of activity do not increase but there is a characteristic continuous locomotion with the body close to the cage floor.

The hyperactivity is greater and of more rapid onset following RbCl than LiCl. This difference was associated with different rates of accumulation of 5-hydroxytryptamine (5-HT), the increases above control values were 46% (Li) and 85% (Rb).

Hyperactivity was inhibited by αmpt (250 mg/kg i.p. 36 h before, plus 150 mg/kg 2 h before, Tc) more effectively following LiCl than RbCl. This combination of αmpt and Tc produced rat brain concentrations of dopamine (DA) significantly below control values in sodium chloride (NaCl) and LiCl pretreated rats but not following RbCl. This suggests that a system which is both 5-HT and DA sensitive is responsible for the hyperactivity.

The increase in brain noradrenaline (NA) concentration following Tc injection was significantly less following RbCl than after NaCl or LiCl. A smaller proportion of NA metabolized by the monoamine oxidase pathway following RbCl treatment could produce this effect. αmpt causes a decrease in rat brain NA concentration which is greater following LiCl or RbCl treatment than after NaCl. This could be due to lithium or rubidium increasing NA 'turnover' rates. All results reported are significant at levels beyond $P = 0.05$.

Previous studies have emphasized the opposite effects of lithium and rubidium on animal activity and brain monoamines. This study shows similar effects on activity when Tc is administered, and both ions increased 5-HT accumulation and NA turnover rates.

The fact that lithium is effective in the treatment of bipolar affective illnesses does perhaps indicate that it has more than one action. Drug-induced hyperactivity in animals has frequently been seen as an analogue to mania, and lithium has often been found to be effective in attenuating those hyperactivity syndromes. The interaction between lithium and Tc is the first to show an activating effect of lithium in animals and might be viewed as a possible experimental model of antidepressant activity. We believe that it is important at this stage to merely show that lithium will produce changes in animal activity and metabolism which could conceivably be analogous to alteration of mood in man. The fact, however, that both caesium and rubidium ions have the same effects suggests that the responses studied are not very specific to lithium. They do, however, also suggest reasons for further clinical studies of rubidium and caesium.

A.J. is a Medical Research Council Scholar. J.P. was supported by the Madge Briggs Memorial Trust.

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Effects of lysergic acid diethylamide on auditory and visual discrimination in the rat

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The effect of LSD on either visual or auditory discrimination was studied in rats matched for discrimination performance. A drug may affect discrimination either by changing stimulus sensitivity or by shifting the criterion for responding. A method of analysis, derived from signal detection theory (SDT), was used to assess independent effects on these two factors.

Twelve adult, male, hooded rats (300 g) were used. Each animal was first trained on a DRL (differential reinforcement of low rate) schedule. A signal indicated the availability of reinforcement. After each reinforced response, the interval to the next signal was varied unpredictably between 9 and 27 s (mean delay 18 s). A response, 9 s or less before the signal, postponed reinforcement for a further 9 s. The schedule yielded a low but constant response-rate. One group of rats ($n = 6$) was trained with a light signal, the second group ($n = 6$) with a tone, and the two groups were matched for discrimination performance. Each rat was trained 45 min per day for 44 days before the experiments, to produce consistent baseline responding. A SDT analysis, described fully elsewhere (Warburton & Brown, 1972), was used to determine two parameters, A', a measure of stimulus sensitivity, and B'', a measure of response bias. The drugs were lysergic acid diethylamide tartrate (Sandoz) and methysergide bimaleate (Sandoz) (a control for peripheral effects). The injection conditions were 0.005, 0.01, 0.05, 0.25 mg/kg LSD, 0.50, 1.00 mg/kg methysergide, and distilled water. The solutions were made up in distilled water and injected i.p. in the volume 1.0 ml/kg. Each rat was tested under each injection condition, with order of injection counterbalanced within each group. Injections were given at 48 h intervals. For statistical analysis results on an injection day were compared with the preceding day's baseline performance.

LSD effects were found to be comparable for the two groups. Total responses were reduced by on average 62.7% ($P < 0.001$) at the highest dose of LSD (0.25 mg/kg). There were no other significant drug effects on response rate. There was a reduction in A' (i.e. a decrease in stimulus sensitivity) at 0.25 ($P < 0.005$) and at 0.05 mg/kg LSD ($P < 0.005$). No other changes in A' under any other injection conditions occurred. The sensitivity changes were not due to changes in response bias (B''), since there were no consistent drug effects on it.

The study demonstrated a reduction in the number of responses at the highest dose of LSD, and independently of this, a decrease in sensitivity to either a tone or a light signal at 0.05 and 0.25 mg/kg LSD. There were no drug effects upon response bias.

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Aversive properties of naloxone in morphine-treated rats

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Rats have been found to reject distinctively flavoured solutions when their consumption on earlier occasions has been followed by poison or radiation-induced illness (Revusky & Garcia, 1970). The poison or radiation is, therefore, defined as an aversive stimulus. This phenomenon has been used to assess the abstinence syndrome precipitated by naloxone in rats chronically treated with morphine.

Rats were allowed access to distilled water between 1 and 2 p.m. daily; no fluid was available at other times. After adaptation to this regime, a saccharin solution was made available instead of distilled water on every third day. Each presentation of saccharin was followed immediately by administration of naloxone hydrochloride or isotonic saline. For 10 days prior to the first presentation of saccharin, and throughout the rest of the experiment, intraperitoneal injections of either morphine hydrochloride or saline were given twice daily (9.30 a.m. and 5.30 p.m.).

In control rats ($n = 8$) receiving saline twice daily, three pairings of saccharin solution (0.1%) with naloxone (10 mg/kg i.p.) failed to induce any reduction in the intake of saccharin. In rats ($n = 8$) receiving morphine (10 mg/kg i.p. twice daily), a single pairing of saccharin with naloxone reduced the mean intake of saccharin from 11.0 ml to 3.0 ml: after three pairings, saccharin intake was 0.6 ml. The intake of distilled water on the days between saccharin presentations was slightly increased ($P < 0.05$), possibly as compensation for the reduced fluid intake when saccharin alone was available. In a second experiment, saccharin was replaced by sodium saccharin, for which rats have a greater preference. The dose of morphine was reduced to 5.0 mg/kg twice daily. A single pairing with naloxone (10 mg/kg i.p.) reduced the mean intake of sodium saccharin solution (0.1%) from 22.0 ml to 9.0 ml ($n = 6$, $P < 0.001$). The intake of distilled water was not suppressed. When the same doses of morphine or naloxone were given separately to different rats, neither drug affected sodium saccharin intake ($n = 6$ in each case). Naloxone was, therefore, an aversive stimulus for rats chronically treated with morphine. Analogous results have been obtained with rhesus monkeys, which learned to press keys to delay infusions of naloxone through implanted venous catheters (Goldberg, Hoffmeister, Schlichting & Wuttke, 1971). Doses of naloxone which were effective aversive stimuli in our experiments also produced signs of mild morphine abstinence, such as defaecation and occasional writhing or weight loss.

The conditioned aversion technique makes possible reliable, objective assessments of abstinence precipitated from low doses of morphine; for example, significant ($P < 0.001$) aversions to sodium saccharin were detected in rats receiving doses of morphine as low as 1.0 mg/kg twice daily. With traditional techniques, experimenters have frequently found it necessary to administer very large doses of morphine in order to produce measurable abstinence signs.

We thank Endo Laboratories for generously providing naloxone.

C.W.T.P. is a Senior Research Fellow of the Mental Health Trust and Research Fund.

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Variations in cGMP and cAMP levels in rat uterine smooth muscle induced by carbachol, PGF$_{2\alpha}$ and changes in ionic composition

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Schultz, Hardman, Schultz, Davis & Sutherland (1973) and Dunham, Haddox & Goldberg (1974) have shown recently that smooth muscle contraction induced by spasmogenic drugs is associated with an increase in cyclic guanosine 3′5′-monophosphate concentration (cGMP).

We have examined the influence of prostaglandin F$_{2\alpha}$ and carbachol (CCh) as well as of different experimental conditions on cAMP and cGMP levels in uterine smooth muscle.

TABLE 1 Ionic content in extracellular solutions and cAMP and cGMP levels in rat myometrium

Test solution				*cAMP mM/mg protein*			*cGMP mM/mg protein*		
Na⁺ mM	*K⁺ mM*	*Ca⁺⁺ mM*	*Ouabain mM*	*Control horn*	*Test horn*	*P <*	*Control horn*	*Test horn*	*P <*
112	4.66	0	0	2.6 ± 0.6 (1)	2.7 ± 0.50	NS	0.23 ± 0.04	0.24 ± 0.10	NS
112	4.66	1.5	1.0	2.9 ± 0.2	3.4 ± 0.30	NS	0.14 ± 0.02	0.14 ± 0.05	NS
16	129	1.5	0	2.9 ± 0.2	4.2 ± 0.30	0.001	0.14 ± 0.02	0.06 ± 0.01	0.001
16	129	1.5	1.0	3.4 ± 0.3*	5.1 ± 0.50	0.001	0.14* ± 0.06	0.12 ± 0.02	NS
16	129	0	0	2.6 ± 0.2	3.3 ± 0.02	0.050	0.22 ± 0.03	0.15 ± 0.01	0.050
87	29	1.5	0	3.3 ± 0.2	3.6 ± 0.20	NS	0.36 ± 0.08	0.26 ± 0.04	NS
87	29	0	0	2.3 ± 0.1	2.2 ± 0.10	NS	0.25 ± 0.03	0.16 ± 0.02	0.020
0**	4.66	1.5	0	3.0 ± 0.2	4.7 ± 0.60	0.020	0.37 ± 0.04	0.35 ± 0.03	NS
0**	4.66	0	0	2.4 ± 0.1	2.7 ± 0.10	NS	0.24 ± 0.03	0.19 ± 0.02	NS

(1) Mean ± standard error.

* 1 mM ouabain added to the control solution.

** Na replaced by Tris Cl.

Uterine horns were excised from virgin Wistar rats pretreated two days before the experiments with 0.5 mg/kg^{-1} day^{-1} of diethylstilboestrol dipropionate. Myometrial strips were prepared by eliminating mucosal and submucosal layers by dissection. The strips were equilibrated before use for at least 90 min in a normal Ringer solution. In each case, one uterine horn was used as a control and the contralateral horn for the experimental manipulation. cAMP and cGMP have been extracted and purified, and cyclic GMP levels determined, by the method of Murad, Manganiello & Vaughan (1971). The cAMP levels were measured by the Gilman technique (1970). The results are expressed in pM/mg of protein (Lowry, Rosebrough, Farr & Randall, 1951). The uterine strips were exposed during 45 s, 1, 2 and 5 min to PGF$_{2\alpha}$ 10^{-5} M, a concentration that produces a maximal contraction. There was no modification of cGMP or cAMP levels under PGF$_{2\alpha}$. CCh was applied to the muscle in concentrations varying from 10^{-6} to 10^{-3} M also for 45 s, 1, 2 and 5 min, without modifying cAMP or cGMP contents.

These results confirm and complete previous findings by Harbon & Clauser (1971). In order to explore further the role of these nucleotides in muscle cell function we have determined cAMP and cGMP levels after 10 min exposures to solutions of different ionic composition (Table 1). The reduction of extracellular Na$^+$ to less than 16 mM increases cAMP levels. This effect may be dependent on a reduction in the intracellular Na$^+$ concentration. The cGMP levels decrease in tissues treated with 129 mM K$^+$, either in the presence or in the absence of Ca^{2+}. This may be related to a stimulation of the Na$^+$-K$^+$-ATPase, since ouabain blocks the reduction of cGMP in high K$^+$ solutions. The effects of low extracellular Ca^{2+} are paradoxical and have to be explored further.

In conclusion cAMP and cGMP do not seem to be involved in the mechanism of contraction of rat myometrium induced by drugs. They may participate in the regulation of some metabolic pathway.

We thank Dr P. Meyer for his helpful advice.

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The effects of cinanserin and phentolamine applied by microiontophoresis in the spinal cord

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Recent studies have shown that conditioning stimulation of the nucleus raphes medianus in the Fluothane anaesthetized rat increases the amplitude of the monosynaptic reflex evoked by dorsal root stimulation (Barasi & Roberts, 1974a). Intravenous L-tryptophan increased the effects of raphe stimulation, indicating that raphe stimulation may activate a pathway releasing 5-hydroxytryptamine (5-HT). More recent studies (Barasi & Roberts, 1974b) have shown that 5-HT applied by microiontophoresis into the ventral horn of the spinal cord increased the amplitude of the motoneurone field potential evoked by antidromic stimulation of the ventral roots. Conditioning stimulation of the nucleus raphes also increased the amplitude of the antidromic field potential. Intravenous (3-4 mg/kg) or iontophoretic (20-100 nA for 5-30 min) applications of cinanserin prevented the effects of raphe stimulation. The specificity of the blocking action of cinanserin remained uncertain, however, particularly as it was noted that a slight increase in dose caused a profound reduction in the amplitude of the unconditioned field potential. We have used noradrenaline as a control agonist to determine the specificity of the action of cinanserin.

Noradrenaline applied with iontophoretic currents between 50 and 100 nA increased the amplitude of the antidromically evoked field potential. Its effects were similar to those of 5-HT but the latency and response duration were longer. We recorded responses to alternate applications of 5-HT and noradrenaline and then superimposed an application of either cinanserin or phentolamine (20-75 nA for 5-20 min). Although both antagonists were capable of blocking responses to both agonists, this occurred with the higher currents of application and was usually but not always accompanied by reduction of the baseline field potential amplitude. The effects of cinanserin on 5-HT were more rapid and longer lasting than its effects on noradrenaline in 11 studies and were similar in two studies. Phentolamine had a greater effect on noradrenaline responses in seven studies and had a similar effect on noradrenaline and 5-HT responses in three studies. Occasionally, a very narrow dose range was identified when the antagonist reduced responses to the agonist without affecting the control agonist responses.

The facilitatory effects of conditioning stimulation of nucleus raphes were recorded during application of the antagonists. It was found that these responses could also be blocked by application of either antagonist but on every occasion the time course of the blockade closely followed the time course of the blockade of 5-HT.

We conclude from these studies that phentolamine and cinanserin can be used to differentiate between responses to noradrenaline and 5-HT in the spinal cord. The results lend support to the postulate that responses of motoneurones to 5-HT and conditioning stimulation of raphe are pharmacologically similar and differ from responses to noradrenaline.

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Amino acid receptors on frog spinal motoneurones

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The isolated hemisected amphibian spinal cord is a convenient preparation for demonstrating the action of neuro-active amino acids (Curtis, Phillis & Watkins, 1961). However, changes in motoneurone activity, as recorded from the ventral roots, reflect not only the direct action of applied substances but also the action of substances on neurones which synapse with motoneurones. Thus, the precise site of action of the applied substances is unknown. To overcome this difficulty, we have blocked regenerative activity with procaine, so allowing the recording of electrotonic propagation in the ventral root to be used as a direct measure of the action of the substances on the motoneurone membrane. With this system L-glutamate and L-aspartate depolarized motoneurones, while γ-aminobutyric acid (GABA) and taurine caused hyperpolarization. The action of GABA was blocked by picrotoxin and bicuculline, but not by strychnine. The action of taurine was blocked by strychnine but not by picrotoxin. Glycine caused a weak depolarization, and neither strychnine nor picrotoxin antagonized this action.

No specific blocking agents are yet available to enable a comparison to be made of the receptors for excitatory amino acids on frog motoneurones with those present on neurones in the mammalian central nervous system. However, a series of excitants covering a wide range of potencies might be expected to show the same order of activity on frog motoneurones as on mammalian spinal neurones if the receptors were similar. Eight such substances (including three new excitants, marked with an asterisk, which have not been previously studied) were tested and the order of excitatory potency was found to be: kainate > N-methyl-D-aspartate ≃ DL-2-amino-4-thiosulphonylbutyrate (thiohomocysteic acid)* ≃ DL-homocysteate > L-glutamate ≃ L-aspartate ≃ 6-hydroxy-2-pyridylalanine (6-H-2PA)* > L-2-amino-3-thiosulphonylpropionate (thiocysteic acid)*. The same order of potency was found for rat spinal interneurones when the substances were administered by microelectrophoresis (Biscoe, Headley, Martin & Watkins, unpublished observations). Moreover, in the frog cord experiments, all the compounds gave a log dose-response plot parallel to that for L-glutamate.

The close similarity between the excitatory amino acid receptors on frog and rat spinal neurones which is apparent from these studies contrasts with the results of structure-activity studies for glutamate agonists on invertebrate preparations, where, for example, kainate and DL-homocysteate have been found to have only weak excitatory actions (Clements & May, 1974). It seems likely that results of further investigations using this amphibian system, including a search for glutamate antagonists, will be relevant to the mammalian central nervous system.

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Sodium and the response of rat descending colon and rat uterus to angiotensin II

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Angiotensin produces a contraction of most isolated smooth muscle preparations (Gross, 1971) and in lower concentrations stimulates trans-epithelial sodium transport (Crocker, 1971). It has been reported that the contractile action of angiotensin on guinea-pig ileum is dependent upon the extracellular sodium concentration (Blair-West, Harding & McKenzie, 1967). In the present study the role of Na^+ has been investigated on the contractile response of rat colon and uterus to angiotensin by altering the Na^+ concentration of the Tyrode solution and by the use of inhibitors of Na^+ movement.

Muscle preparations, from Wistar rats, were suspended in Tyrode solution and gassed with air. Contractions were measured at the maximum sustained deviation from resting tension using concentrations of acetylcholine and angiotensin which produced responses approximately the same size as 50% maximum acetylcholine response.

When the Na^+ concentration of the Tyrode solution was varied from the normal concentration of 137 mmol the responses of rat colon to acetylcholine and to angiotensin changed similarly being reduced by 12% at 120 mmol and 50% at 68.5 mmol Na^+ and reduced by 30% at 171 mmol and 60% at 205.5 mmol Na^+. At high concentrations of ouabain (1.0 mmol) there was equal reduction of responses to acetylcholine and to angiotensin of both rat colon (37%) and rat uterus (57%). After 0.1 mmol ethacrynic acid, the isometric responses of rat colon to acetylcholine were reduced by 15.4 ± 0.7% while the angiotensin response was reduced by 42.9 ± 5.3% ($n = 6$, p 0.001). With rat uterus, it was necessary to record responses isotonically (1 g load), there was equal reduction (39%) of responses to acetylcholine and to angiotensin. However, with 3 g load, there was a significantly greater reduction ($n = 7$, P 0.01) of the angiotensin response (60.0 ± 3.6%) compared with the acetylcholine response (35.8 ± 4.4%).

Therefore, a preferential reduction of the angiotensin response of uterus was observed only under conditions of high energy expenditure. Since it has been reported that ethacrynic acid inhibits cellular energy production (Epstein, 1972) it appears that its effect on uterus might be due to this mechanism rather than an effect on Na^+ movement. This conclusion is supported by the severe inhibition of all contractile responses observed during exposure of rat uterus to higher concentrations of ethacrynic acid (0.5 mmol). It is also consistent with our previous observations that the angiotensin response of these tissues involves an ATP-dependent step (Crocker & Wilson, 1975, Wilson, Crocker & Willavoys, 1974). The present findings, however, do not support a specific role for Na^+ in the interaction of angiotensin with these tissues.

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A study of tetramethylenedisulphotetramine (TETS) and related compounds as antagonists of presynaptic inhibition and microiontophoretically applied γ-aminobutyric acid (GABA) and glycine in the rat cuneate nucleus

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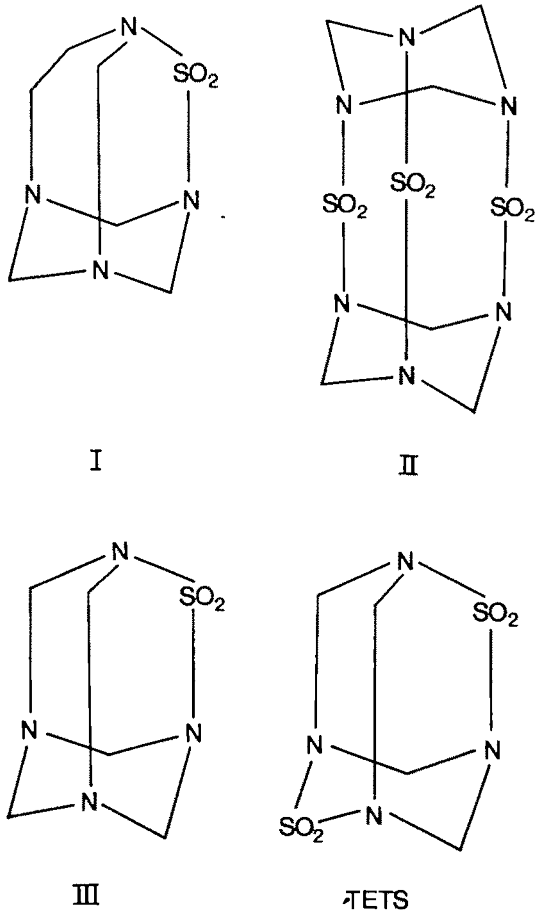
TETS is an extremely potent convulsant that antagonizes the actions of GABA at the rat superior cervical ganglion (Bowery, Brown & Collins, 1975) and at the crustacean neuromuscular junction (Large, 1975). However, these preparations are insensitive to glycine and so give no indication of the specificity of TETS as a GABA antagonist. We have therefore examined the specificity of TETS and its effect on presynaptic inhibition in the rat cuneate nucleus. In addition, three other structurally related compounds, (Figure 1), have been examined.

Experiments were performed on 14 rats anaesthetized with urethane and prepared as described by Hill & Miller (1974). Presynaptic inhibition was estimated from the amplitude of the P-wave component of the cuneate field potential produced by stimulation of the appropriate forepaw (Anderson, Eccles, Schmidt &



I II III TETS

Figure 1.

Yokota, 1964). To examine the specificity of TETS, GABA and glycine were applied to single cuneate neurones by microiontophoresis (Hill & Simmonds, 1973) and the depression of firing so produced examined before and after the administration of TETS. Due to the low solubility of TETS (<300 μg/ml saturated aqueous solution) it was not possible to apply it by microiontophoresis.

Compounds I, II and III had no effect on presynaptic inhibition when given intravenously in doses up to 2 mg/kg or when superfused over the medulla in physiological saline (500 μg ml^{-1} h^{-1}). However, doses of TETS as low as 20 μg/kg effectively antagonized presynaptic inhibition as measured by either test.

TETS given by intravenous infusion (100 μg ml^{-1} h^{-1}) was found to antagonize the effect of microiontophoretically applied GABA but this was always accompanied by a parallel antagonism of glycine and so cannot be considered to be specific. However, its actions may not be completely non-specific as it does not antagonize the action of carbachol on the superior cervical ganglion (Bowery *et al.*, 1975).

This work was supported by a grant from the M.R.C.

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Some neuropharmacological properties of the new non-barbiturate hypnotic etomidate (R (+)-ethyl-1-(α-methyl-benzyl) imidazole-5-carboxylate)

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Etomidate is a novel short acting hypnotic of great potency and low toxicity. The drug has two optical isomers but only the (+) isomer possesses the hypnotic properties (Janssen, Niemegeers, Schellekens & Lenaerts, 1971). Experiments have been performed in an attempt to elucidate its mode of action.

Both isomers were administered to rats and mice by a variety of routes. Mice were injected intracerebroventricularly by the method of Brittain & Handley (1967) and the ED$_{100}$ for ten minutes loss of righting reflex was determined. For the (+) isomer this was 67.0 ± 9.2 μg, but the (−) was inactive at doses up to 500 μg.

Using the technique of microiontophoresis (Hill & Simmonds, 1973) GABA, glycine and both isomers of etomidate were applied alternately from multi-barrelled micropipettes onto single neurones in the cuneate nuclei of urethane anaesthetized rats. Neurones were found to be readily depressed by GABA, glycine and both isomers of etomidate and, in terms of the applying currents used, the isomers were equipotent and approximately half as active as either GABA or glycine. In the same rats intravenous administration of the (+) isomer (0.25-1.0 mg/kg) produced a pronounced increase in the proportion of slow wave activity in the EEG with concurrent depression of single neurone firing. Topical superfusion of the medulla with solutions of (+)-etomidate in physiological saline (5×10^{-3} M) also significantly depressed neuronal firing. (−)-Etomidate (2 mg/kg; 5×10^{-3} M) was inactive in both of these tests.

In vitro tests of the activity of etomidate were also performed. Respiration of isolated rat brain tissue and mitochondria (Kerkut, Rick & Taberner, 1972) was not depressed by either isomer at concentrations (0.01 mM) in excess of those reported to occur in rat brain following the administration of hypnotic doses (Heykants, 1974). The active uptake of [^{3}H]-GABA and [^{14}C]-glutamate into rat brain slices was also studied (Balcar & Johnston, 1972). This uptake was found to be ouabain sensitive and temperature dependent but was not affected by either isomer of etomidate at concentrations up to 0.2 mM.

It can therefore be concluded that (+)-etomidate is an effective hypnotic at concentrations which do not affect essential tissue metabolism or uptake of putative transmitter substances. The results of the microiontophoretic experiments would suggest that the differing potencies of the two isomers are more likely to be due to the relative ease with which they gain access to their site of action rather than to an intrinsic difference in potency.

We would like to thank Janssen Pharmaceutica for the etomidate.

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Preliminary pharmacological study of N,2-dimethyl,2-(p-aminophenyl) succinimide

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Aminoglutethimide is the p-aminophenyl derivative of the hypnotic drug glutethimide. It was available as an anti-epileptic compound until 1966, when it was withdrawn because of adverse drug reactions including several endocrine effects. These latter effects were associated largely with an inhibition by the drug of adrenal steroid biosynthesis. Because of this activity, aminoglutethimide is being evaluated in patients with excessive cortisol and aldosterone production (Hughes & Burley, 1970). We have synthesized p-aminophenyl derivatives of a number of substituted glutarimides and succinimides with a view to determine their pharmacological activity and potential therapeutic value. The present report describes some pharmacological properties of one of these compounds, N,2-dimethyl,2-(p-aminophenyl) succinimide, (aminomethsuximide).

The compound was synthesized by the nitration and subsequent reduction of N,2-dimethyl, 2-phenylsuccinimide, (methsuximide). Chemical structure and purity were confirmed by elemental analysis, i.r., u.v. and n.m.r. analysis and mass spectrometry.

Anticonvulsant activity of aminomethsuximide and methsuximide (both at 50 mg/kg i.p.) was determined in male mice and rats. Both compounds exhibited anti-leptazol activity. The response of mice, in groups of six, to leptazol (100 mg/kg s.c.) was expressed as a seizure severity score and the area under the response/time (to 6 h) curve was taken to represent the overall effect. On this basis, aminomethsuximide and methsuximide reduced seizure activity by 25.9 and 35.4% respectively and the times of maximum effect were at 1 and 4 h respectively for these compounds. In rats (groups of ten) leptazol was infused i.v. at a constant rate to determine the minimum dose inducing clonic convulsion (Orloff, Williams & Pfeiffer, 1949). Anticonvulsant activity, expressed as percentage increase in threshold dose of leptazol over controls, was 20% and 73% respectively, 1 h after administration of aminomethsuximide and methsuximide. At the above doses, these succinimides did not protect mice against maximal electroshock. The intermediate compound, N,2-dimethyl,2-(p-nitrophenyl) succinimide (50 mg/kg i.p.) was inactive in all of these tests.

In male rats (160 g; six per group) receiving three daily doses of aminomethsuximide (50 mg/kg i.p.) there was a significant ($P < 0.001$) increase in adrenal weight (mg of adrenal pairs: 21.3 ± 2.05 treated, 16.8 ± 1.83 control) and a significant ($P < 0.001$) decrease in adrenal corticosterone concentration (μg/50 mg adrenal: 3.57 ± 1.09 treated, 7.16 ± 0.71 control). Thin-layer chromatography of adrenal extracts indicated that there was a higher concentration of cholesterol in glands from aminomethsuximide-treated rats than controls. Histological examination of the adrenals from the treated animals showed the presence of extensive fatty vacuolation of the zona fasciculata. On the same dose schedule, methsuximide did not produce any of these effects in rats. However, aminoglutethimide (150 mg/kg i.p.) given to rats for three days caused a fall in corticosterone content of a similar order to that following administration of aminomethsuximide and produced similar changes in adrenal histology. It is concluded that aminomethsuximide has an inhibitory action on adrenal steroidogenesis and that further investigation of the endocrine effects of the compound would be worthwhile.

We are grateful to Parke-Davis & Co., Pontypool, Gwent, for a sample of methsuximide and for a research grant.

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Estimates of catecholamine turnover rates in individual hypothalamic nuclei of the rat by use of alpha-methyl-para-tyrosine

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Administration of alpha-methyl-para-tyrosine (αMpT) or its methyl ester has been shown to inhibit tyrosine hydroxylase, the rate limiting step in catecholamine synthesis (Spector, Sjoerdsma, & Udenfriend, 1965). The disappearance of endogenous catecholamines after inhibition of tyrosine hydroxylase has been used to estimate the rate of turnover of catecholamines in brain and other tissues. However, there has been some debate concerning the proper method of administration and the validity of the estimates of turnover rates because the falls in catecholamine levels are multiphasic (Iversen & Glowinski, 1966; Costa & Neff, 1966; Javoy & Glowinski, 1971; Doteuchi, Wang & Costa, 1974).

The purpose of our study was to measure the turnover rates of catecholamines in various hypothalamic nuclei. The recent availability of microdissection techniques and highly sensitive biochemical methods for assay of biogenic amines has made it possible to localize more specifically alterations in the effects of catecholamine metabolism and thereby to define more precisely the functional significance of the different nuclei.

Adult, male rats (Zivic-Miller), 200-250 g were given various doses of L-αMpT or DL-αMpT methyl ester (Sigma), and killed by decapitation at intervals up to 5 h, after initial drug administration. The brains were rapidly removed, frozen, and microdissection was performed by the method of Palkovits, Brownstein, Saavedra & Axelrod (1974). Dopamine and other catecholamines (predominently norepinephrine) were then measured by a sensitive radiometric assay (Coyle & Henry, 1973).

Our findings were: (1) Intravenous administration of DL-αMpT methyl ester, 200 mg/kg, produced no inhibition of catecholamine synthesis. (2) After 200 mg/kg i.v. of L-αMpT, there was a biphasic decline in the dopamine and norepinephrine levels in the median eminence (ME), ventromedian nucleus (n v m), and medial forebrain bundle (MFB), with the more rapid decline occurring during the first hour. (3) L-αMpT, 200 mg/kg i.v., or DL-αMpT methyl ester, 400 mg/kg i.p., were inadequate to prevent levels of catecholamines from increasing after 3 h; therefore, a second dose was required at that time to maintain inhibition of tyrosine hydroxylase. (4) There appeared to be no significant difference in the turnover rates of catecholamines after administration of either L-αMpT, 200 mg/kg i.v., or DL-αMpT, 400 mg/kg i.p. (5) After 400 mg/kg DL-αMpT methyl ester i.p. at 0 and 3 h, the rate constants for the 1-5 h interval for dopamine in ME, n v m, and MFB were respectively: 0.154 ± 0.058, 0.176 ± 0.068, and 0.0964 ± 0.066 (h^{-1}, mean ± standard deviation, $n = 10$); and for norepinephrine for these same regions: 0.0633 ± 0.0631, 0.138 ± 0.089, and 0.142 ± 0.056.

The results of this study suggest that: (1) it is possible to estimate relative rates of turnover of catecholamines in small discrete areas of brain by inhibition of their synthesis; (2) large and frequent doses of the inhibitory agent must be administered to ensure effective blockade of synthesis, (3) because of the multiphasic nature of the decline in amine content, caution must be exercised in interpreting these results in absolute terms. This method may be of value, however, in identifying changes in catecholamine turnover and, by implication, functional activity of such nuclei after various forcings.

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Amphetamine and apomorphine responses in the rat after lesion of mesolimbic or striatal dopamine neurones

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While low doses of amphetamine increase locomotor activity in the rat, higher doses produce stereotyped behaviour. There is evidence that release of dopamine (DA) in the brain is responsible for these actions of amphetamine. Dopamine is principally localized in two systems of neurons, the nigrostriatal pathway, and the mesolimbic dopaminergic system arising in the A 10 group of neurons and terminating in the nucleus accumbens, olfactory tubercle and frontal cortex. Selective lesions have been made of one or other of these systems using stereotaxic injection of the selective neurotoxic agent 6-hydroxydopamine (6-OHDA). The behavioural effects elicited by low and high doses of d-amphetamine and by the dopamine agonist apomorphine were examined in these two preparations. Locomotor activity was measured in photocell cages, and the intensity of the stereotyped behaviour was evaluated with a 1-6 rating scale.

Eight μg of 6-OHDA injected into the nucleus accumbens septi (NAS) in a volume of 2 μl reduced the DA content of the NAS to 7% of control and that in the olfactory tubercle to 22% of control when brains were assayed 18 days later. Striatal DA was only depleted by 17%. When brains were assayed 90-100 days after the lesion the DA concentration in the NAS had recovered to values significantly greater (21% of control), although there was no similar recovery of amine levels in the other regions studied. Injections of 8 μg of 6-OHDA into the caudate nucleus reduced caudate DA levels by 51% when assays were performed 90-100 days later, but did not affect DA in the nucleus accumbens or olfactory tubercle. The caudate lesion attenuated the intense stereotyped behaviour produced by 5 mg/kg (i.p.) of d-amphetamine, but did not alter the locomotor stimulation produced by 1.5 mg/kg (i.p.) of d-amphetamine. The dopamine agonist apomorphine (1 mg/kg i.p.) produced more intense stereotyped behaviour in the caudate lesioned animals, which may be attributed to supersensitivity of the denervated striatal DA receptors. By contrast the NAS lesion severely attenuated the locomotor response to 1.5 mg/kg (i.p.) of d-amphetamine, whereas the locomotor response to apomorphine (0.1-1.0 mg/kg i.p.) was greatly enhanced. The locomotor stimulant effect of apomorphine was blocked by the DA antagonist pimozide (0.5 mg/kg). The stereotyped behaviour produced by 5 mg/kg of d-amphetamine was not attenuated. These behavioural effects of the NAS lesion were maximal 14-22 days after the lesion. Thereafter there was a gradual recovery, with an increase in the locomotor response to d-amphetamine (1.5 mg/kg) and a corresponding decline in the locomotor response to apomorphine. The behavioural changes correlate with the recovery of DA content in the NAS.

The NAS lesioned animal offers a convenient *in vivo* model for studying the effects of dopamine agonists and antagonists on mesolimbic DA receptors (Kelly, Miller & Neumeyer, D.1, this meeting).

Responses of cortical pyramidal tract cells to amantadine and amphetamine after depletion of central catecholamines

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Amantadine is an anti-Parkinsonian drug for which there is evidence for three possible modes of action: (a) a direct stimulation of postsynaptic receptors; (b) An amphetamine-like action to release presynaptic catecholamines; (c) Inhibition of reuptake of released catecholamines.

From previous experiments we concluded that (c) did not contribute to the depression of neurones seen with iontophoretically applied amantadine (Stone & Bailey, 1975). The present experiments were an attempt to assess the contribution of (a) and (b) to the depressions produced by amantadine.

Male hooded Wistar rats weighing 250-300 g were pretreated 24 h before the acute experiments with one of the following: 1 ml/kg of 0.9% saline; 1 ml/kg Tween 80; 200 mg/kg alpha-methylparatyrosine methyl ester in saline; 10 mg/kg reserpine suspended in Tween 80.

For the acute experiments animals were anaesthetized with 1-1.25 g/kg urethane, and the frontoparietal cerebral cortex was exposed. To compare responses in normal and amine-depleted animals only one type of cell was studied. These were pyramidal tract cells with antidromic latencies of less than 3.0 ms. Five-barrelled micropipettes filled with 200 mM solutions of the various drugs were used for microiontophoresis. Ejecting currents of 60 nA were applied for 15 s in all cases. Three parameters of the neuronal response were measured to allow comparison of the normal and treated animals. These were *h*, the maximum change of firing rate produced, *t*, the time taken to reach that maximum, and *d*, the total duration of the response.

The reduction of amine levels was confirmed by fluorimetric estimation of noradrenaline and dopamine in the cortex.

Dopamine responses were unchanged in the amine depleted animals when compared to controls. Responses to amphetamine and amantadine were significantly reduced in amplitude *h*, but were not abolished. The parameters *t* and *d* were unchanged.

Failure to abolish amphetamine responses by amine depletion has been reported in the cerebellum (Kostopoulos & Yarborough, 1974) and caudate nucleus (Feltz & de Champlain, 1972) and seems to indicate an important difference in the action of amphetamine in these areas and in the brain stem (Boakes, Bradley & Candy, 1972).

The present experiments suggest that on pyramidal tract cells amphetamine and amantadine do not act solely by releasing stored catecholamines.

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Effect of dopamine-β-hydroxylase inhibitors and centrally administered noradrenaline on (+)-amphetamine anorexia in mice.

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Whilst the finding that α-methyl p-tyrosine antagonizes (+)-amphetamine anorexia in mice (Abdallah, 1971) implied possible involvement of catecholamines in the production of (+)-amphetamine anorexia in this species, resolution of the individual function of dopamine and noradrenaline in this capacity has not been attempted. Consequently, we have undertaken a preliminary study of the interactions between (+)-amphetamine, noradrenaline and centrally acting dopamine-β-hydroxylase inhibitors in mice.

Male albino mice of an ICI strain, weighing 20-25 g were housed in groups of 8 at an environmental temperature of 25 ± 1°C and trained over 10 days to adapt to a daily 3 h period of a 41B cube diet consumption. Water was given *ad libitum*. In trained mice, (+)-amphetamine injected subcutaneously, 15 min before feeding, produced a dose related depression of food intake during the first hour of feeding, and 2 mg/kg which produced a submaximal response, was subsequently used as the standard anorexic dose.

Intracerebroventricular (icv) injection of noradrenaline (4 and 8 μg in 5 μl saline) 25 min before feeding, potentiated (+)-amphetamine anorexia significantly ($P < 0.01$) only at the higher dose level. At doses of 250 and 500 mg/kg both disulfiram given orally 3 h before food and sodium diethyldithiocarbamate (DDC) given intraperitoneally 2 h before food markedly potentiated (+)-amphetamine anorexia ($P < 0.001$, $P < 0.001$). Neither noradrenaline nor the dopamine-β-hydroxylase inhibitors significantly influenced feeding when given alone at these dose levels. However, the potentiation of (+)-amphetamine anorexia by DDC was partially, though significantly reversed by noradrenaline (4 and 8 μg icv ($P < 0.05$, $P < 0.001$), although noradrenaline and DDC in combination did not significantly alter feeding compared with saline controls.

The potentiation of (+)-amphetamine anorexia by dopamine-β-hydroxylase inhibitors in mice differs from the antagonism seen in rats (Frey & Schulz, 1973) and this discrepancy may reflect species variation in the mechanism of action of (+)-amphetamine and/or DDC. In addition, although the potentiation of (+)-amphetamine anorexia by icv noradrenaline appears consistent with an involvement of noradrenaline in the mediation of (+)-amphetamine anorexia, this finding is difficult to reconcile with the potentiation of (+)-amphetamine anorexia brought about by DDC and disulfiram and the partial reversal by icv noradrenaline of the intense anorexia following DDC pretreatment. Nevertheless, both sets of results remain compatible with an influence of the noradrenergic component of the central action of (+)-amphetamine on the anorexia produced by this drug in mice although the exact nature of this role requires further study.

However, the conflicting nature of these results leads us to suspect that the mechanisms underlying (+)-amphetamine anorexia in mice cannot be interpreted solely in terms of noradrenergic systems and in spite of the lack of direct evidence, when considered in conjunction with the results of Abdallah (1971), they appear to indicate the possibility of a significant dopaminergic component in the production of anorexia by (+)-amphetamine in the mouse.

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The mechanism of the effect of dopamine-β-hydroxylase inhibitor FLA-63 on the L-DOPA reversal of reserpine akinesia

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The effects of dopamine-β-hydroxylase inhibitors (DBHI) on behaviour are thought to result from their ability to deplete cerebral noradrenaline (NA) (Svensson & Waldeck, 1969). The pattern of motor activity produced by L-DOPA in reserpinized animals is attenuated by pretreatment with the DBHI bis-(4-methyl-1-homopiperazinylthiocarbonyl)disulphide (FLA-63) (Ahlenius & Engel, 1971; Marsden, Dolphin, Duvoisin, Jenner & Tansy, 1974).

We have therefore compared the effect of pre-treatment with FLA-63 on the reversal of reserpine akinesia by L-DOPA with changes in brain catecholamine content.

Pre-treatment with i.p. FLA-63 caused a dose-dependent increase in locomotor activity in

Table 1 Effect of L-DOPA (200 mg/kg i.p. plus the peripheral decarboxylase inhibitor α-methyldopa hydrazine 25 mg/kg i.p.) on motor activity and whole brain catecholamine content in mice reserpinized (10 mg/kg i.p.) 18-24 hours previously. Animals are given either saline or FLA-63 one hour prior to L-DOPA.

Pretreatment	*Animex Counts 1st hour after L-DOPA*	*DA ng/g 45 mins after L-DOPA*	*NA ng/g 45 mins after L-DOPA*
Saline (0.1 ml i.p.)	2520.5 ± 294.4 (45)	972.8 ± 117.6 (12)	52.1 ± 12.4 (14)
FLA-63 (10-25 mg/kg i.p.)	2729.3 ± 322.9 (24)	1667.9 ± 208.8^c (8)	31.8 ± 11.4 (8)
FLA-63 (40-50 mg/kg i p.)	3704.6 ± 400.0^b (14)	2166.8 ± 372.9^c (11)	49.2 ± 8.9 (11)
	2nd and 3rd hour after L-DOPA	*120 mins after L-DOPA*	*120 mins after L-DOPA*
Saline (0.1 ml i.p.)	10641.5 ± 505.6 (41)	2109.9 ± 199.1 (10)	67.5 ± 17.4 (12)
FLA-63 (10-25 mg/kg i.p.)	7210.3 ± 713.0^d (23)	3792.4 ± 595.8^d (8)	15.0 ± 2.6^a (8)
FLA-63 (40-50 mg/kg i.p.)	5134.9 ± 1108.1^d (9)	3026.3 ± 453.4^a (11)	19.3 ± 1.9^a (9)

The results are given ± s.e.mean and the number of experiments is indicated in brackets. Significant differences from control values are given by superscripts $^aP < 0.05$, $^bP < 0.01$, $^cP < 0.005$, $^dP < 0.001$.

the first hour after L-DOPA compared with control animals; this was associated with a dose-dependent increase in DA but no significant change in the NA content of whole brain at 45 min after L-DOPA administration. Pretreatment with FLA-63 caused a dose-dependent suppression of locomotor activity compared with control animals in the second and third hours after L-DOPA; this was associated with a significant decrease in brain NA in the presence of persistent and significant elevations in brain DA, as shown in Table 1.

Further experiments to exclude possible stressful effects of FLA-63 (Moore & Thornburg, 1971) showed that FLA-63 administered in solution by oral intubation did not produce any hypermotility in the first hour after L-DOPA (the Animex counts for high and low dosage levels were 73 and 64% of controls respectively). This was associated with significantly increased DA and decreased NA. In the second time period there was no significant difference between the two routes of FLA-63 administration in either the effect on motor activity or the CA levels. Furthermore, neither corticosterone (3 mg/kg s.c.) nor β-methasone (0.1 mg/kg s.c.) given 15 min before L-DOPA had any significant effect on L-DOPA induced motor activity.

Thus, it appears that the effects of FLA-63, whether administered orally or intraperitoneally, in reducing L-DOPA induced locomotor activity in the reserpinized animal are due primarily to the extent of reduction in cerebral NA, and cannot be explained by stressful effects.

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Dopaminergic and cholinergic interactions in the caudate nucleus in relation to the induction of sleep in the cat

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The onset of sleep is characterized by the appearance of spindles (8-12 Hz activity) in the electroencephalogram. Electrical stimulation of the caudate nucleus elicits similar spindles (Buchwald, Wyers, Okuma & Heuser, 1961). Using a push-pull cannula localized areas of the brain can be perfused and stimulated (Philippu, Przuntek & Roensberg, 1973). The present study was carried out to examine the effects of perfusions of dopamine and acetylcholine on spontaneous and electrically induced spindles.

Experiments were carried out on *encéphale isolé* cats. A bi-polar stimulating electrode was placed stereotaxically in the head of the caudate nucleus. The anode of the electrode was a tube which was also used for perfusions. Throughout the experiment the nucleus was perfused with artificial cerebro-spinal fluid (c.s.f., 150 μl/min at 38° C). Drugs were added to the c.s.f. as required, and perfused for 15 min intervals, alternating with c.s.f. alone. Cortical activity was recorded from frontal, parietal and occipital lobes and was correlated with behavioural signs of waking or sleeping.

Perfusions of acetylcholine (5.5×10^{-2} or 5.5×10^{-1} M) plus the anticholinesterase physostigmine (2×10^{-9} M) inhibited spontaneous spindling and this effect was atropine sensitive. In some cats perfusions of dopamine (5×10^{-6} M) plus the monoamine oxidase inhibitor tranylcypromine (2×10^{-8} M) increased spontaneous spindling. However, injections of 0.2 μl of dopamine (9.5×10^{-4} mol or 9.5×10^{-3} mol) into the caudate nucleus invariably induced ipsilateral frontal spindles which, in most cats, developed into behavioural and electroencephalographic sleep.

When the caudate nucleus was stimulated electrically, a strength of stimulus (pulse width 0.5 ms) was selected which produced ipsilateral frontal spindles. Repeated stimulation at 5 s intervals for up to 1 min produced similar responses and in some cats induced sleep. Perfusion of c.s.f. containing dopamine plus tranylcypromine potentiated the effects of stimulation. This was observed as a spread of spindles into the contra-lateral frontal, and non-frontal cortices. In some animals, stimulation which did not produce sleep during control perfusions of c.s.f., induced sleep during perfusions of dopamine plus tranylcypromine. Perfusion of acetylcholine plus physostigmine reduced or abolished the spindling induced by electrical stimulation.

These results suggest that dopaminergic and cholinergic mechanisms in the caudate nucleus are involved in the control of cortical spindling.

P.E.K. is an M.R.C. Research Fellow.

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Hypotensive action of α-methyldopamine

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It is generally agreed that α-methyldopa exerts a hypotensive action via interference with the sympathetic nervous system, and that α-methyldopa must undergo decarboxylation to form α-methyldopamine and α-methylnoradrenaline in order to exert this effect (Davis, Drain, Horlington, Lazare & Urbanska, 1963). Carlsson & Lindqvist (1962) suggested that these decarboxylation products of α-methyldopa may take over the functions of monoamines in the brain, and Day & Rand (1964) extended this hypothesis to peripheral adrenergic nerves.

Most of the studies on α-methyldopa have concentrated on α-methylnoradrenaline as a false transmitter in the periphery although recent evidence suggests a central locus of action of α-methyldopa (Henning & Van Zwieten, 1968; Finch & Haeusler, 1973).

In this study we have examined the activity of α-methyldopamine which has been largely neglected, although Farmer (1965) has shown that it impairs peripheral sympathetic nerve activity and Heise & Kroneberg (1973) have demonstrated a central hypotensive action when α-methyldopamine was infused intraventricularly into the chloralose anaesthetized cat.

α-Methyldopamine (50-200 μg i.c.v.) caused a dose-related fall in blood pressure in conscious spontaneous hypertensive rats. Pretreatment with intraventricular 6-hydroxydopamine (3 x 250 μg) prevented this hypotensive effect of α-methyldopamine (150 μg i.c.v.). Intraventricular administration of phentolamine (200 μg) or desmethylimipramine (200 μg), but not haloperidol (0.5 mg/kg i.p.) prevented the hypotensive action of α-methyldopamine (150 μg i.c.v.). Pretreatment with U-14, 624 (200 mg/kg i.p.), a selective central dopamine-β-hydroxylase inhibitor also prevented the hypotensive effect of α-methyldopamine (150 μ i.c.v.).

In the chloralose anaesthetized cat pressor responses elicited by stimulation of the midbrain reticular formation (Finch & Haeusler, 1973) were reduced after intraventricular injection of α-methyldopamine (1 mg) and completely abolished with 5 mg.

Intraventricular administration of α-methylnoradrenaline (20-100 μg) to the chloralose anaesthetized cat caused dose related pressor responses.

Intravenous α-methyldopamine was considerably less potent than noradrenaline as a pressor agent in the pithed rat, but noradrenaline and α-methylnoradrenaline were found to be equipotent.

These results suggest that the hypotensive effect of α-methyldopamine may be mediated via central action of α-methylnoradrenaline on α-adrenoceptors. As a prerequisite α-methyldopamine must be taken up into adrenergic neurones to produce this effect. However, α-methylnoradrenaline (i.c.v.) in chloralose-anaesthetized cats caused a dose related rise in blood pressure which does not support this hypothesis.

Since α-methyldopamine is considerably less potent than noradrenaline as a pressor agent in the pithed rat, it is possible that this might reflect partially, a false transmitter action of α-methyldopa in the periphery, but the present observations still support a central hypotensive action.

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The potentiation of certain effects of amphetamine by inhibitors of prostaglandin synthesis

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Sever & Trelinski (1974) have shown that indomethacin, a prostaglandin synthesis inhibitor (PGSI), potentiates the hyperthermic effect of amphetamine in the rat.

We now report other studies in which we show that other PGSIs (mefenamic acid, flufenamic acid and antipyrine) also potentiate the amphetamine-induced hyperthermia in rats, and alter the pattern of behavioural stimulation in rats and mice.

Female Wistar rats (150-200 g body weight) were used, and the hyperthermic effect of amphetamine measured as described previously (Caldwell, Sever & Trelinski, 1974). Drug induced behavioural changes were measured both in rats (housed singly) and in female T.O. mice (20-25 g body weight) in groups of six (Sever, Caldwell & Williams, in preparation). The PGSIs were administered orally 1 h prior to the intraperitoneal injection of D-amphetamine (5 mg/kg). Control animals were treated with normal saline.

A dose related potentiation of amphetamine induced hyperthermia was caused by indomethacin (5-15 mg/kg) mefenamic acid (50-200 mg/kg), flufenamic acid (50-200 mg/kg) and antipyrine (50-150 mg/kg). However, paracetamol (50-300 mg/kg) had no effect on the intensity of the hyperthermia. All of the PGSIs altered the pattern of behavioural stimulation seen after amphetamine, but there was species variation in the nature of the changes. In the rat, the gross locomotor stimulation was decreased, but stereotyped behaviour was unchanged, while in the mouse, the converse occurred.

In further experiments, rats were given 6-hydroxydopamine (100 mg/kg i.v.) to destroy catecholamine-containing nerve terminals in the periphery. Forty-eight hours later, they were given PGSIs or saline, followed by amphetamine, as before. No hyperthermia was observed in either test or control group.

Indomethacin, mefenamic acid, flufenamic acid and antipyrine which are inhibitors of peripheral PG synthesis (Flower, 1974) all produced a potentiation of the amphetamine hyperthermia. However, this was not seen in animals pretreated with 6-hydroxydopamine. Paracetamol, which only inhibits brain PG synthesis (Flower, 1974), is without effect on the amphetamine hyperthermia, this hyperthermia depending on intact peripheral noradrenergic neurones (Caldwell *et al.*, 1974). It has been suggested that inhibition of PG synthesis abolishes the PG control over noradrenaline release (Smith, 1972), leading to a potentiation of the hyperthermic effect of amphetamine (Sever & Trelinski, 1974) and the results here are consistent with this view.

The pretreatment of animals with PGSIs followed by amphetamine also leads to changes in the pattern of behavioural stimulation due to amphetamine. Since the central stimulant action of amphetamine is apparently due to catecholamine release in the brain (Costa & Groppetti,

1970) it may be that inhibition of PG synthesis in the brain affects the actions of amphetamine in the same way as in the periphery.

We are grateful to Professor R.L. Smith and Professor R.T. Williams, FRS, for their interest in this work.

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Dissociation of bacterial pyrexia from prostaglandin E activity

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Prostaglandins of the E series (PGE's) elevate body temperature in a number of species and have been implicated as hypothalamic mediators of fever (Milton & Wendlandt, 1971; Feldberg & Saxena, 1971). Whereas PGE_1 infused into the hypothalamus of adult fowls (Nisticò & Marley, 1973) or young chicks (Artunkal & Marley, 1974) elevated body temperature at a thermoneutral ambient temperature, it was found to be markedly hypothermic when infused into the hypothalamus of young chicks at an ambient temperature (Ta) below thermoneutrality, viz 16°C (Artunkal & Marley, 1974), 16°C is not a severe thermal load for chicks of this age (Marley & Stephenson, 1975). Both the hyperthermic and hypothermic effects of PGE_1 in young chicks were potentiated by indomethacin (Artunkal & Marley, 1974), a prostaglandin synthetase and dehydrogenase inhibitor (Ferreira, Moncada & Vane, 1971; Vane, 1971; Flower, 1974).

Present experiments exclude the possibility that this potentiation was solely due to inhibition of prostaglandin dehydrogenase, since similar potentiation was obtained after pretreatment with 5,8,11,14-eicosatetraynoic acid (TYA), 3.4 μmol/100 g, i.v., 30 min previously; TYA is an arachidonic acid analogue which selectively inhibits prostaglandin synthetase (Flower, 1974). The International Pyrogen reference preparation, *Shigella dysenteriae* (1 μg), infused into the hypothalamus of chicks consistently elevated body temperature by 0.5°-2.0°C after a delay of 1-2 h at ambient temperatures of 31° and 16°C, effects that were not potentiated by indomethacin (1.4 μmol/100 g., i.v., 30 min previously).

Comparison of the effects of PGE_1 with those of *Shigella dysenteriae* yielded two important differences: (1) PGE_1 was hypothermic at a TA of 16°C whereas *Shigella dysenteriae* elevated body temperature; (2) the effects of *Shigella dysenteriae* on body temperature, unlike those of PGE_1, were not potentiated by indomethacin. Nor could the hyperthermic effects of *Shigella dysenteriae* be attributed to PGE_2 since at a Ta of 16°C, PGE_2 (14.3 nmol) infused into the hypothalamus, lowered body temperature up to 4.75°C.

The results demonstrate that prostaglandins of the E series are not consistently hyperthermic in all species. Thus, apart from the results in young chicks, hypothermic effects of intraventricular injection of PGE_1 and PGE_2 (each 2 μg) have been demonstrated over a wide range of ambient temperatures in the *Echidna, Tachyglossus aculeatus* (Baird, Hales & Lang, 1974). Additionally, fever evoked in young chicks by bacterial pyrogen can be dissociated from the effects of PGE_1 or PGE_2 in at least two important ways.

Our thanks are due to Dr John Pike of Upjohn Chemical Co. for PGE_1, to Merck, Sharp & Dohme Ltd. for indomethacin, to Dr D.R. Bangham of The National Institute for Medical Research for *Shigella dysenteriae* and to Roche Ltd. for TYA.

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Substrate and strain-dependent differences in the development of monoamine oxidase in the rat brain

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Physicochemical studies and *in vitro* experiments using different enzyme inhibitors have overwhelmingly pointed to the existence of different forms of the enzyme monoamine oxidase (MAO) (for review, see Sandler & Youdim, 1972). Their presence and independent function *in vivo* is more difficult to ascertain. It has been indicated in experiments on rats treated with progesterone in which the increase in adrenal MAO caused by this steroid was significantly different when different

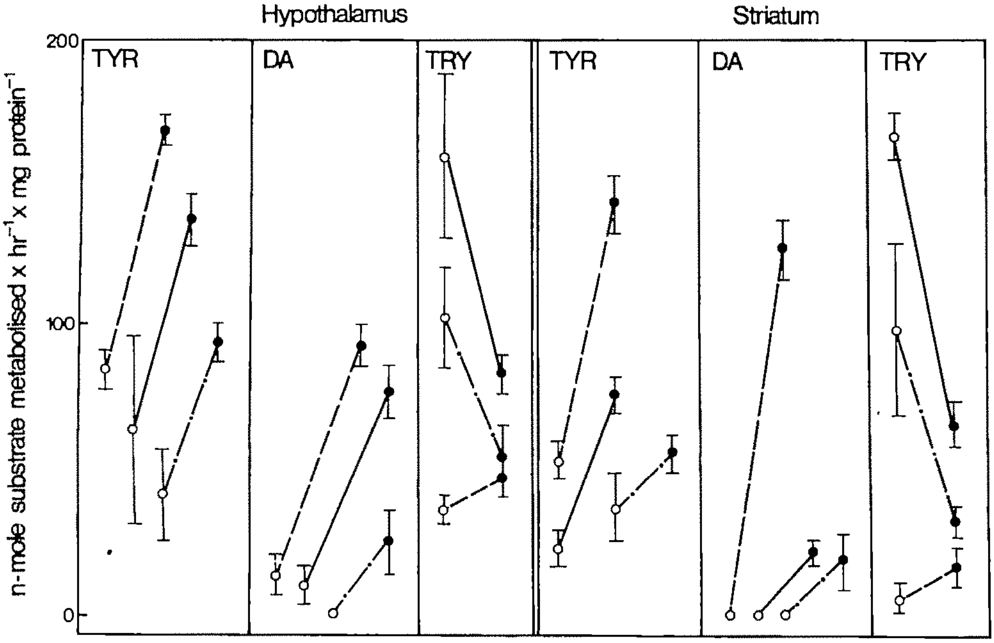

Figure 1 MAO activity in the hypothalamus and striatum of five day old (○) and 20 day old (●) rats. – – –: Porton rats. ——: Wistar rats, bred at Tuck's. · · · · ·: Wistar rats, bred from Tuck stock at Babraham. TYR = tyramine; DA = dopamine; TRY = tryptamine. Mean value ± s.e. of the mean (n = 5).

substrates were used (Youdim, Holzbauer & Woods, 1974). To further elucidate the problem experiments are in progress to examine whether the rate of the age dependent changes in brain MAO activity (Karki, Kuntzman & Brodie, 1962) varies when different substrates (kynuramine, K; tyramine, TYR; dopamine, DA; tryptamine, TRY; and 5-hydroxytryptamine, 5-HT) are used. The brain regions studied were the hypothalamus, striatum, septum and cerebellum. Litter mate rats (5, 10, 20, 40 and 80 days old) of different strains were used. Figure 1 shows examples of MAO-activity in the hypothalamus and striatum of 5 (○) and 20 (●) day old rats of three different colonies. With TYR and DA as substrate MAO activity was higher in both brain regions of the older rats of all three colonies. This was also the case with tryptamine in rats from the Porton strain, although to a lesser degree. In contrast, both brain regions of the five day old rats of the two Wistar colonies showed a considerably higher activity towards tryptamine than those of the 20 day old rats. In a group of five day old 'hooded rats' hypothalamic MAO activity towards tryptamine was only about one half that found in 30 day old rats of the same colony. However, the caudate nucleus of the five day old hooded rats exhibited five times more MAO activity towards tryptamine than that of the 30 day old rats. MAO activity towards DA was absent in the striatum of all five day old rats so far tested. The results obtained with other brain regions and peripheral tissues (adrenal glands, heart and liver) also showed differences in the rates of development of MAO activity depending on the substrate used.

Thus it appears possible that the postulated multiple forms of MAO do not develop at the same rate in the growing rat. This differential development may reflect the maturation of different mitochondria concerned with the metabolism of biogenic amines (Youdim, 1974).

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The specificity of the binding of ^{3}H-5-hydroxytryptamine (^{3}H-5-HT) to butanol extracts of rat brain

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In a previous communication to the Society (Godwin & Sneddon, 1974) we described the binding peak of ^{3}H-5-HT (5×10^{-7} M) eluted from an LH20 Sephadex column by a discontinuous chloroform-methanol (CM) gradient. This peak was found to be highly sensitive to the degree of hydration of the extract and was reduced by lysergic acid diethylamide (LSD) (3.2×10^{-7} M-2.5×10^{-6} M) in a concentration-dependent manner.

The following experiments examine the selectivity of this technique and measure the specificity of the 5-HT binding.

Atropine and amphetamine (both at 5×10^{-7} M) had no effect on the peak of eluted ^{3}H-5-HT when preincubated with the extract whereas 5×10^{-5} M amphetamine and tryptamine, and reserpine (4×10^{-6} M) and pargilline (5×10^{-7} M) all significantly reduced the proportion of the label eluted in the 5-HT binding peak.

Following double labelling of the extract with ^{14}C-Ach (10^{-6} M) and ^{3}H-5-HT (5×10^{-7} M), the elution profile of the 5-HT was unaltered (see figure). The ^{14}C-label was distributed between two peaks, one eluting in the C/CM 15 : 1 interface (peak *A*) and the other at the beginning of elution with CM 4 : 1 (peak *B*).

Preincubation with tubocurarine (5×10^{-7} M) had no effect on the ^{3}H-5-HT elution but greatly reduced the ^{14}C-label in peak *B*, proportionately increasing the level in peak *A*.

We have also studied extracts of that part of the rat diaphragm rich in motor nerve endplates (Hebb, Krnjević & Silver, 1964) and found that after double labelling and subsequent chromatography, there is a considerable increase in the proportion of ^{14}C-label in peak *B* (the overall

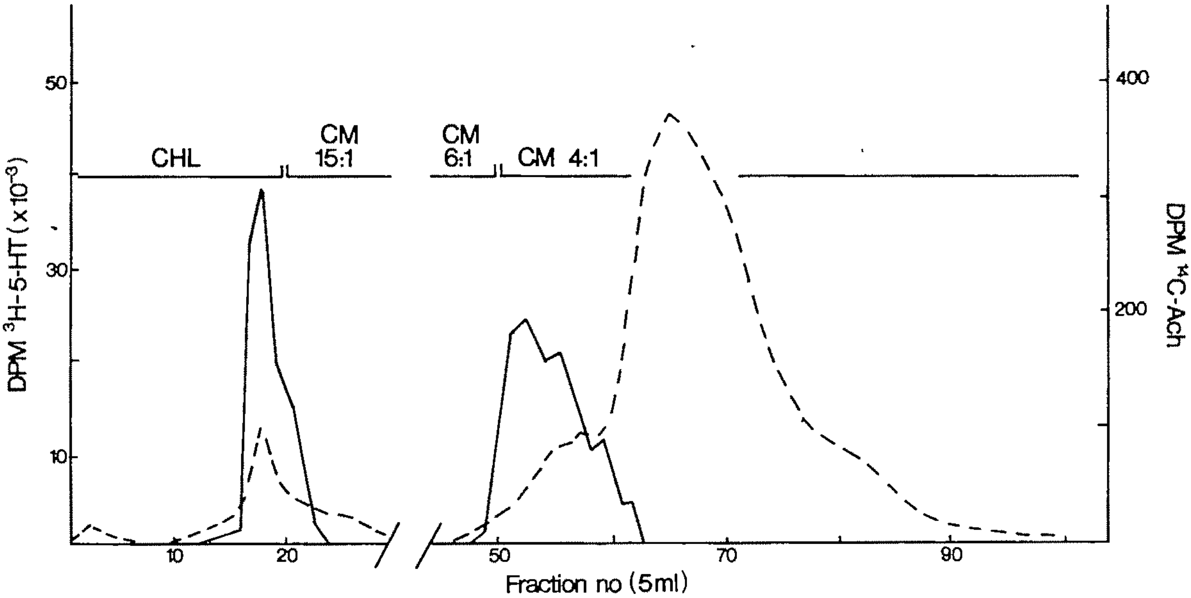

Figure 1 Double-label (—— = ^{3}H-5-HT), – – – = ^{14}C-Ach) binding to butanol extract of rat brain and elution on an LH20 Sephadex column (2.6 x 32 cm) using 100 ml Chl, 50 ml each of CM 15 · 1, CM 10 : 1, CM 6 : 1, and then 300 ml CM 4 : 1.

ACh-binding increase notwithstanding). No binding of ^{3}H-5-HT was observed, the ^{3}H-label appearing in the region where free ^{3}H-5-HT is known to elute.

Further drug studies designed to examine the specificity of these interactions are in progress.

S.G. is an M.R.C. Scholar.

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Effect of propranolol treatment on the development of DOCA/saline hypertension in rats

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The mechanism whereby propranolol lowers arterial blood pressure in man is uncertain and attempts to reproduce this action in laboratory animals have produced conflicting results. In rats with established DOCA/saline hypertension neither Farmer & Levy (1968) nor Conway (1974) detected any antihypertensive effect of β-adrenoceptor blocking doses of propranolol, whilst Dusting & Rand (1974) reported a marked fall in blood pressure in their experiments using low twice daily doses (0.2 mg kg^{-1} day^{-1}) of propranolol. Similarly, inconsistent results have been published concerning the influence of β-adrenoceptor blockade on the development of hypertension in rats. Weiss, Lundgren & Folkow (1974) reported that both propranolol and the cardioselective β-adrenoceptor blocker H93/26 markedly reduced the development of hypertension in spontaneously hypertensive rats (SHR) and Conway (1974) has made a similar observation with propranolol and ICI 66082 in SHR and DOCA/saline hypertensive rats. However, Lundgren (1974) found that propranolol did not influence the development of renal (unilateral renal artery constriction) hypertension in rats and Frohlich (1974) observed a similar lack of effect of sotalol treatment on the development of hypertension in SHR.

The present study was designed to test the effects of a range of propranolol doses on the development of DOCA/saline hypertension in rats. Male Wistar rats (60-90 g) were made hypertensive by implanting a 25 mg DOCA pellet subcutaneously, left nephrectomy and substitution of 1% saline for drinking water for the 14 days following the operation. Systolic blood pressures were measured by the tail cuff method, twice weekly, for the first eight weeks after operation and thereafter weekly. Propranolol treatment was started two days after operation and continued for five weeks. Daily intraperitoneal doses of propranolol used were 25, 10, 2, 0.2 and 0.02 (mg $kg^{-1}day^{-1}$).

In untreated DOCA/saline rats the systolic blood pressure rose steeply during the first one to two weeks following operation from average values of 125 mm Hg to 160 mm Hg. Thereafter the blood pressure rose much more gradually reaching a level of 190-200 mm Hg by week 12. None of the dose-regimens of propranolol used produced any marked effect on the initial rapidly developing phase of the hypertension but at each dose level the secondary slowly developing phase was markedly reduced. The 10 mg $kg^{-1}day^{-1}$ regimen of propranolol was slightly more effective than the 25 mg $kg^{-1}day^{-1}$ dose level but the three lower dose levels each produced a similar reduction in the development of hypertension as did the 10 mg $kg^{-1}day^{-1}$ level. When the propranolol treatments were discontinued the blood pressure of each group of treated animals remained at or only slightly above the treatment levels for the next seven weeks.

Parallel experiments were performed using either α-methyldopa (100 mg $kg^{-1}day^{-1}$ i.p.) or pargyline (10 mg $kg^{-1}day^{-1}$ i.p.) instead of propranolol. Both treatments markedly reduced the development of hypertension both in the rapid early and slow secondary phase of the disease.

The results suggest that propranolol can markedly reduce the development of hypertension in DOCA/saline treated rats. Previously reported failures to do this may possibly be accounted for by too high doses of propranolol and/or too short a period of treatment. α-Methyldopa and pargyline produced a similar effect to propranolol on the slow secondary phase of the hypertension and in addition markedly reduced the rapidly developing phase.

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Cyclic adenosine-3',5'-monophosphate in cerebrospinal fluid

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Recently Dascombe & Milton (1975) have reported raised levels of cyclic adenosine-3',5'-monophosphate (cAMP) in the cerebrospinal fluid (c.s.f.) of the unanaesthetized cat during bacterial pyrogen fever. In the present work experiments have been conducted to determine the effects of heat and cold stress on c.s.f. levels of cAMP, the effects of intravenous cAMP on body temperature, and the passage of intravenous cAMP across the blood/c.s.f. barrier.

The method described by Feldberg, Gupta, Milton & Wendlandt (1973) was used to obtain samples of c.s.f. from the unanaesthetized cat. The cAMP content of the c.s.f. was measured after ethanol deproteinization by competitive binding assay (Brown, Albano, Ekins, Sgherzi & Tampion, 1971; Gilman, 1970). The animals were individually caged and unrestrained, rectal temperature was monitored continuously.

Animals were cold stressed by exposure to an ambient temperature of 0 ± 2° C for three hours. During exposure to cold, body temperature increased and was associated with vasoconstriction, a crouched posture and shivering. Levels of cAMP in c.s.f. removed during and after this period were not significantly different ($P > 0.2$) from control levels (ambient temperature 25 ± 1° C).

Exposure to an ambient temperature of 45 ± 1° C for 3.5 h caused the body temperature of the cats to rise 2.45 ± 0.30° C and was associated with vasodilatation, panting and stretching out. Upon cessation of heat exposure body temperature fell rapidly to control levels. Levels of cAMP in c.s.f. during and after heat stress were not significantly different ($P > 0.1$) from control.

cAMP (0.1-10 mg/kg) injected intravenously in cats at an ambient temperature of 22 ± 2° C caused a dose-related, rapid increase in the amount of cAMP assayed in c.s.f. High doses (5 mg and 10 mg/kg) of cAMP, but not lower doses (0.1 mg and 1 mg/kg), produced a significant ($P < 0.01$) fall in rectal temperature, which began 2-3 min after injection and reached a maximum in about 18 minutes. The hypothermia was associated with ear skin vasodilatation, and in one animal in response to cAMP 10 mg/kg, polypnoea and sweating from the paw pads.

Intravenous injections of ^{3}H-cAMP were followed by a rapid rise in ^{3}H-cAMP levels in the c.s.f. showing that the exogenous nucleotide was passing from the blood into the c.s.f.

The level of cAMP in c.s.f. may therefore be raised as a result of increased levels of cAMP in the blood; as these results show, the nucleotide rapidly enters c.s.f. from the blood. The raised levels of cAMP recently reported following i.v. bacterial pyrogen could possibly result from raised levels of cAMP released peripherally by the pyrogen. The raised levels of cAMP reported during bacterial pyrogen-induced fever are not, however, considered a consequence of either raised body temperature or active thermoregulatory processes.

M.J.D. is in receipt of a Medical Research Council Scholarship. This research was supported by a grant from the Medical Research Council.

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Cyclic AMP in developing chick brain: changes with ischaemia and catecholamine administration

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Cyclic AMP has been implicated in the functioning of the central nervous system, and there is evidence indicating that cyclic nucleotides are involved in the control of cell growth and differentiation (for review, see Drummond, 1973). The concentration of cyclic AMP in rat brain has been reported to increase throughout development (Schmidt, Palmer, Dettbaru & Robison, 1970; Ebadi, Weiss & Costa, 1971), but the method of sacrifice used in these studies does not eliminate rapid post-mortem changes in the nucleotide (Nahorski & Rogers, 1973).

We have measured the concentration of cyclic AMP in the cerebral hemispheres of chicks during their neonatal development using a method whereby brain tissue is removed and frozen by a freeze-blowing technique which largely eliminates post-mortem changes (Nahorski & Rogers, 1973). Cyclic AMP was assayed by the protein binding saturation method of Brown, Albano, Ekins, Sgherzi & Tampion (1971). The cyclic AMP content of freeze blown chick cerebral hemispheres was found to decrease during neonatal development, from 12.5 ± 0.8 p moles/mg protein in one day old chicks to 3.0 ± 0.01 p moles/mg protein in 28 day old chickens. However, in chicks killed by decapitation there was a rapid increase in cerebral cyclic AMP. This post-mortem rise in the nucleotide concentration increased with age. One minute of ischaemia induced an increase of 23.5 p moles/mg protein in the one day old chick and 48 p moles/mg protein in the 28 day old bird compared with the appropriate controls. It is possible, therefore, that the discrepancy between the developmental changes in cyclic AMP obtained in the present study and those obtained in the rat by Schmidt *et al.* (1970) may be due to age dependent post-mortem hypoxic increases in brain cyclic AMP.

In the neonate chick with its immature blood-brain barrier, the systemic administration of β-adrenoceptor agonists has been shown to increase the concentration of cyclic AMP in chick cerebral hemispheres *in vivo* (Edwards, Nahorski & Rogers, 1974). In the present study chicks of varying ages were injected intravenously with isoprenaline (5 μmoles/kg) and were killed by freeze-blowing 3 min later. The isoprenaline-induced increase in cyclic AMP was maximal in three day old chicks (+19.7 p moles/mg protein) but diminished with age (+3.3 p moles/mg protein in 28 day old birds).

^{3}H-noradrenaline was given intravenously to chicks aged 2-28 days, and its penetration into the brain was determined. Brain levels of ^{3}H-noradrenaline at 28 days of age were only 19% of those at two days of age. It is probable, therefore, that the decrease in the *in vivo* cyclic AMP response to isoprenaline with age is due to the development of a blood-brain barrier to catecholamines rather than a change in receptor sensitivity. *In vitro* studies to further substantiate these results are now in progress.

Financial support from the Medical Research Council is gratefully acknowledged.

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Antinociceptive activity in mice after central injections of α- and β-adrenoceptor antagonists

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Recent studies have demonstrated that narcotic agonist and partial agonist analgesic agents interact in a specific way with certain putative central transmitters: thus, the intracerebroventricular (ICV) injection of 5-hydroxytryptamine (5-HT) potentiates whilst the ICV injection of noradrenaline (NA) attenuates the antinociceptive activity of these agents in experimental animals (Sewell & Spencer, 1974). The present study sets out to characterize more fully the nature of this attenuation by ICV-administered NA.

Nociceptive sensitivity was measured repeatedly at fixed intervals in ICI-derived albino mice weighing 18-22 g, using the tail-immersion method described in the earlier report. It had already been demonstrated that the centrally-acting α-sympathomimetic agent clonidine possessed antinociceptive activity in experimental animals (Schmitt, Le Douarec & Petillot, 1974), and this has been confirmed by the present authors using the tail-immersion test in mice. However, when clonidine was given by ICV injection (0.5 μg/animal), it possessed only marginal antinociceptive activity, and furthermore substantially attenuated the antinociceptive effect of morphine (3 mg/kg) when given concurrently. The α-adrenergic antagonist phentolamine (10 μg/animal, ICV) exhibited antinociceptive activity when given alone and significantly potentiated the activity of a subcutaneously administered concurrent dose of morphine (3 mg/kg) or pentazocine (15 mg/kg). The antinociceptive activity of subcutaneously administered clonidine (300 μg/kg) on the other hand was significantly antagonized by phentolamine (5 μg/animal, ICV).

The β-adrenergic antagonist propranolol possesses no antinociceptive activity when peripherally administered (Cicero, Meyer & Smithloff, 1974). However, when given alone, propranolol (10 μg/animal, ICV) exhibited some antinociceptive effect but it did not potentiate the activity of concurrent subcutaneous doses of morphine (2.5 mg/kg), pentazocine (15 mg/kg) or clonidine (300 μg/kg, s.c.).

Cicero *et al.* (1974) have shown previously that peripherally-administered α-adrenergic antagonists enhance the antinociceptive effects of morphine in mice. The present study shows this is also true when these agents are restricted to the central nervous system by virtue of their ICV route of injection; also, that the antinociceptive activity of a partial agonist is also enhanced, whilst that of clonidine is attenuated. Whilst the enhancement of the action of narcotic agonists and partial agonists by α-adrenergic antagonists is entirely consistent with the previously demonstrated effects of ICV NA, the significance of the antinociceptive effect of ICV propranolol requires further study.

We are indebted to Messrs Boehringer & Sohn, Ingelheim, for generous financial assistance.

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The uptake of mescaline by rat brain synaptosomes

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It has previously been reported that single cortical neurones can respond with excitation or depression to microelectrophoretically applied mescaline (Bevan, Bradshaw, Roberts & Szabadi, 1974). It has also been reported that these neuronal responses can be potentiated by the tricyclic antidepressant desipramine (Bevan, Bradshaw & Szabadi, 1975). It is of interest to know whether this potentiation could be due to the blockade of the uptake of mescaline into presynaptic terminals. Iversen (1967) has reported that mescaline has a very low affinity for noradrenaline uptake mechanisms in the periphery, but there are no data concerning the uptake of mescaline into brain tissue.

I have therefore examined the uptake of (^{14}C)-mescaline (specific activity 5.2 mCi/mmole, New England Nuclear Corpn) and, for the purposes of comparison, (^{14}C)-(−)-noradrenaline (specific activity 5.0 mCi/mmole, Radiochemical Centre Ltd.) into synaptosomes prepared from rat cerebral cortex according to the method of Thornburg & Moore (1973). After incubation, the mescaline or noradrenaline content of each sample was assessed by liquid scintillation spectrometry, and the protein content determined using the method of Lowry, Rosebrough, Farr & Randall (1951). The uptake of mescaline or noradrenaline was thus expressed as pmoles/mg protein for each sample. All values are expressed as mean ± s.e.

In agreement with earlier reports, for example, Horn, Coyle & Snyder (1971), the accumulation of noradrenaline was found to be a temperature-dependent process ($K_m = 0.628 \pm 0.033$ μM, $V_m = 192.3 \pm 7.4$ pmoles/mg protein). Mescaline was also accumulated by an active uptake process ($K_m = 1.24 \pm 0.27$ μM, $V_m = 26.34 \pm 8.02$ pmoles/mg protein); however, for equivalent concentrations, the uptake of mescaline was much lower than that of noradrenaline. For example, at a concentration of 0.628 μM, the K_m of noradrenaline, the uptake of mescaline was 10% the uptake of noradrenaline.

The effect of desipramine on the uptake of noradrenaline and on the uptake of mescaline was then examined. Desipramine competitively inhibited the uptake of noradrenaline (K_i desipramine = 0.0499 ± 0.0052 μM). However, desipramine, within a concentration range 0.05-5 μM, did not affect the uptake of mescaline.

The effect of mescaline on the uptake of noradrenaline was also examined. Mescaline was found to inhibit the active accumulation of noradrenaline in a non-competititive manner (K_i mescaline = 10.548 ± 0.697 μM).

The data presented here suggest that the active accumulation of mescaline into synaptosomes from the cerebral cortex is not brought about via the noradrenaline uptake mechanism. Furthermore, since the uptake of mescaline was not affected by desipramine, uptake blockade cannot explain the potentiation of neuronal responses to mescaline by desipramine.

Paul Bevan is the Burmah Oil Research Fellow of the Mental Health Trust and Research Fund.

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Depleting effects of reserpine on intracellular catecholamines in rat coeliac-mesenteric ganglion

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Since the introduction of formol-induced fluorescence methodology for the histochemical demonstration of biogenic amines these have been studied in sympathetic ganglia not only in adrenergic post-ganglionic neurones but also in small intensely fluorescent (sif) cells (Eranko & Harkonen, 1963) present in these ganglia. Sif cells have been equated (Grillo, Jacobs & Comroe, 1974) with the small granular cells revealed by electron microscopy in sympathetic ganglia (Williams & Palay, 1969). Recently, Lever, Lu, Presley & Santer (1974) have demonstrated chromaffin-positive (CH+) small cells with a similar distribution to sif cells in a variety of sympathetic ganglia employing glutaraldehyde tissue fixation prior to dichromate treatment. Current speculation suggests that small cells in sympathetic ganglia may have an inhibitory effect on post-ganglionic transmission acting either interneuronally or in a local endocrine capacity.

The aim of the present investigation was to test the lability of sif and CH+ cells to reserpine in terms of their specific fluorophore emission and chromaffin positivity respectively in adult rat coeliac-mesenteric ganglia. Two reserpine dosage schedules were applied (a) 5 mg/kg i.p. 6 h before sacrifice and (b) 5 mg/kg i.p. at 36, 24 and 12 h before the animals were killed. Control animals were correspondingly injected with saline. After single injections of reserpine there was a statistically significant ($P < 0.001$) reduction in the % of chromaffin-positive and a statistically significant ($P < 0.001$) increase in the % of chromaffin-negative small cells compared with controls. Although after single injections of reserpine specific amine fluorophore was not detected in principal ganglionic neurones, no obvious reduction in fluorophore emission from sif cells was apparent. However, after prolonged reserpinization (3 x 5 mg/kg) not only was there a highly significant reduction in the % of chromaffin-positive small cells but fluorimetric measurements from sif cell cytoplasmic areas were significantly ($P < 0.001$) lower (by a factor of 2) than from comparable cells areas in control animals.

Our results are more definitive than those of Van Orden, Burke, Geyer & Lodoen (1970) who reported 'slight variable reduction in fluorescence intensity' from sif cells following two reserpine injections.

The investigation was supported by a grant from the Medical Research Council.

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The effects of nicotinic and muscarinic agonist drugs on the release of catecholamines from the isolated perfused adrenal glands of the dog

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Douglas & Poisner (1965) found that, in isolated perfused adrenal glands from cats, pilocarpine released almost solely adrenaline, whereas nicotine released about equal proportions of adrenaline and noradrenaline.

Working with anaesthetized animals, Critchley, Ungar & Welburn (1973) found that specific sensory stimuli in the cat caused reflex release of either mainly adrenaline or mainly noradrenaline. However, when similar sensory stimuli were applied to dogs the release occurred without a change in the ratio of adrenaline to noradrenaline. We decided to extend the work of Douglas & Poisner to the dog, using the more specific nitocinic and muscarinic agonists that are now available. We wanted to see whether cats differ from dogs in their selective responses to cholinergic agonists as well as in their physiological responses to sensory stimuli.

The glands were perfused retrogradely with oxygenated Lockes solution at 37° C through the adrenolumbar vein at a flow of 1 ml/minute. The effluent was collected for 1 min periods and the catecholamines estimated by the trihydroxyindole method (Vendsalü, 1960).

The glands were stimulated by changing the perfusate to one containing either m hydroxyphenylpropyl trimethyl ammonium iodide (10^{-8}-10^{-5} M) (Barlow & Franks, 1971) or acetyl β methyl choline (10^{-8}-10^{-6} M) as nicotinic and muscarinic agonists respectively.

In 14 tests on nine glands to which acetyl β methyl choline was given, the resting output of catecholamines was 390 ± 80 ng/min of which 22 ± 1% was noradrenaline. During stimulation the output rose by 1.4 to 8.6 fold and the increment contained 27 ± 3% noradrenaline.

In 17 tests on nine glands to which m hydroxyphenyl propyl trimethyl-ammonium iodide was given the resting output of catecholamines was 350 ± 80 ng/min of which 21 ± 2% was noradrenaline. During stimulation the output rose by 1.2 to 26 fold and the increment contained 24 ± 2% noradrenaline.

However, with cat glands we find that acetyl β methyl choline preferentially releases adrenaline while the increments with our nicotinic agonist contains a greater proportion of noradrenaline.

We conclude that in the dog, as in the cat, both nicotinic and muscarinic agonists release catecholamines from the adrenal gland. In contrast to the situation in the cat, the ratio of noradrenaline to adrenaline released in the dog changes little over a wide range of intensity of stimulation.

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Characterization of the β-adrenoceptors of guinea-pig tracheobronchial, skeletal and cardiac muscle

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Certain selective β_2-adrenoceptor stimulants given systemically produce effective bronchodilatation in man with minimal cardiovascular effects (Beumer, 1971; Legge, Gaddie & Palmer, 1971) but enhancement of physiological tremor is obvious in many patients. Studies in anaesthetized cats indicate that the tremor-inducing and bronchodilating actions of these drugs are mediated through similar β-adrenoceptors (Bowman & Nott, 1970). We now wish to report results of experiments designed to characterize the β-adrenoceptors in guinea-pig tracheobronchial, skeletal and cardiac muscles by using selective and non-selective β-adrenoceptor agonists and antagonists.

Experiments were performed using preparations of tracheal segments (James, 1969) and soleus muscles in pentobarbitone-anaesthetized guinea-pigs. Cumulative dose-response curves were determined for the agonists, (-)-isoprenaline being used as a reference drug in each experiment. (-)-Isoprenaline (0.001-3.0 μg/kg i.v.) had a similar potency in decreasing tracheal segment pressure, in decreasing the tension of submaximal tetanus of a soleus muscle and in increasing heart rate. However, the selective β_2-adrenoceptor agonists salbutamol (0.01-1000 μg/kg i.v.), terbutaline (0.1-1000 μg/kg i.v.) and fenoterol (0.01-300 μg/kg i.v.) were each less effective on heart rate than on tracheal pressure or muscle tension. The results are summarized in Table 1.

Propranolol (0.01-10 mg/kg i.v.) blocked responses to (-)-isoprenaline in the same dose range in all three tissues. Practolol (0.1-10.0 mg/kg i.v.) was more effective in blocking heart rate responses to isoprenaline than tracheal segment or soleus muscle responses. H35/25 (dl-erythro-4′-methyl-α(1-isopropylaminoethyl)-benzylalcohol hydrochloride) (0.1-10.0 mg/kg i.v.) was more effective in blocking tracheal segment and soleus muscle responses than heart rate responses to (-)-isoprenaline (Table 1).

No evidence was obtained with the drugs used to indicate that the β-adrenoceptors in guinea-pig tracheobronchial muscle differed from those in skeletal muscle.

Table 1 Activity of selective and non-selective β-adrenoceptor agonists and antagonists on tracheobronchial, skeletal and cardiac muscle of the anaesthetized guinea-pig

AGONISTS	*Equipotent dose (95% confidence limits) for*		
	Decrease in intratracheal segment pressure	*Decrease in tension of submaximal tetanus*	*Increase in heart rate*
(−)-Isoprenaline	1.0	1.0	1.0
Fenoterol	7.8	5.9	158.9
	(4.7-13.2)	(3.7-9.5)	(121.4-207.9)
Salbutamol	9.5	9.4	73.9
	(5.9-15.4)	(5.6-15.7)	(48.5-112.5)
Terbutaline	28.7	34.4	257.2
	(20.5-40.1)	(22.1-53.7)	(172.8-382.9)

ANTAGONISTS	*Dose (mg/kg i.v.) (95% confidence limits) for 50% inhibition of (−)-isoprenaline responses on*		
	Tracheal segment	*Soleus muscle*	*Heart rate*
Propranolol	0.05	0.04	0.06
	(0.02-0.14)	(0.01-0.09)	(0.04-0.09)
Practolol	8.2	12.8	0.9
	(2.8-24.0)	(4.8-34.2)	(0.5-1.9)
H35/25	0.2	0.3	>10
	(0.1-0.4)	(0.2-0.4)	(17-47% inhibition at 10 mg/kg; lethal at 30 mg/kg)

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Inhibition of constrictor responses of the rabbit ear artery by a mixture of oxytetracycline and ascorbic acid

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Powis (1973) reported marked potentiation of constrictor responses of the isolated perfused rabbit ear artery by oxytetracycline, 10^{-4} mol/l. In attempting to confirm this finding, a brand of oxytetracycline was used which consists of a mixture of oxytetracycline and ascorbic acid powder. When this is made up to a solution containing oxytetracycline 10^{-4} mol/l, ascorbic acid is present at a concentration of 1.3 x 10^{-3} mol/l. Addition of this mixture to the perfusing fluid of the artery had no appreciable effect on its pH, but caused a progressive and marked reduction in responses to noradrenaline so that after 30 min perfusion average responses (rise in perfusion pressure) in six experiments to 5, 10 and 20 ng of noradrenaline were reduced from 25 ± standard error 3 to 11 ± 3, from 40 ± 6 to 15 ± 3 and from 57 ± 9 to 23 ± 6 mm Hg respectively. ($P < 0.01$—paired t test—in each case)—Figure 1. Addition of the oxytetracycline alone caused a small, non-significant increase in responses (Figure 1).

In a further series of six experiments, ascorbic acid alone at a concentration of 1.1 x 10^{-3} mol/l caused only slight reduction of responses to noradrenaline. Responses to 5, 10 and 20 ng of noradrenaline were reduced from 29 ± 6 to 25 ± 5 ($P < 0.05$), from 42 ± 8 to 37 ± 7 ($P < 0.02$) and from 60 ± 13 to 53 ± 11 mm Hg ($P > 0.05$) after 30 min perfusion.

Constrictor responses to histamine (0.5, 1.0, 2.0 µg) showed a similar marked depression in the presence of the mixture of oxytetracycline and ascorbic acid. This depressant action of the mixture on arterial constrictor responses appears to be another example of the unexpected effects of tetracyclines when mixed with stabilizing agents (Sulkowski & Haserick, 1964).

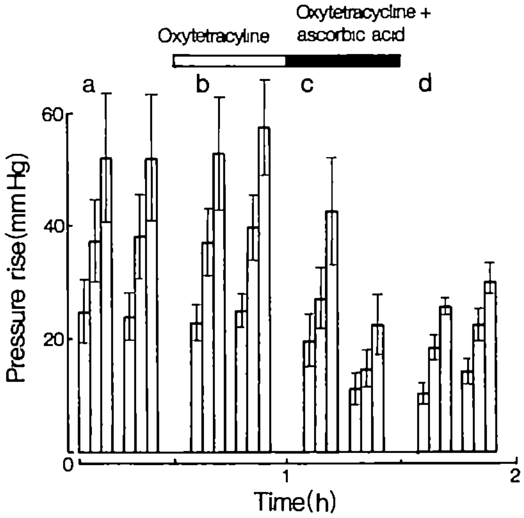

Figure 1 Effect on responses (rise in perfusion pressure) to noradrenaline of oxytetracycline, 10^{-4} mol/l and oxytetracycline 10^{-4} mol/l plus ascorbic acid 1.1 x 10^{-3} mol/l in the perfusate. Graded responses to noradrenaline, 5, 10 and 20 ng are shown. Results are means with standard errors from six experiments: (a) under control conditions, (b) with oxytetracycline present, (c) with the mixture present and (d) after a return to the control perfusate.

We would like to thank Mr R.G.P. Watson for helping with some of the experiments and Pfizer Ltd. for supplying oxytetracycline.

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Adrenergic blockade and the pulmonary pressor response to lactic acid

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Vasoconstriction in the lung is an important protective response. Its mechanism in relation to drugs and hypoxia has been studied by others.

We find that the pulmonary vascular bed in the sheep responds to small doses of 4 ml 0.75 molar lactic acid with a 50% or greater increase in mean pulmonary artery pressure (P$\bar{p}$a).. The lactic acid is injected into the jugular vein (J.V.) and the response follows in nine seconds. The P$\bar{p}$a returns to near the previous level in four minutes. The test can be repeated many times provided sodium bicarbonate is given to maintain blood pH at or above 7.3. The sheep are anaesthetized with halothane and ventilated. Pressures are recorded continuously from the pulmonary artery and left ventricle.

Previous experiments have shown that the rise in Ppa is not due to an increase in flow or elevation of left atrial pressure but the change in Ppa reflects a change in pulmonary vascular resistance. It is of interest that lactic acid is a vasoconstrictor in the pulmonary vascular bed and a vasodilator in the skeletal muscular bed.

The injection of 10 mg phentolamine into the J.V. results 5 to 10 min later in marked reduction or abolition of the response to lactic acid.

Infusion of phenoxybenzamine, 30 mg in 200 ml of saline in 30 min, results 75 min later in the disappearance of the pressor response to lactic acid.

Reserpine 2.5 mg prevented the response after a variable time of the order of two hours. Atropine (12-17 mg) had no effect on the response.

Thus α-adrenoceptor blockade prevents the expected increase in P$\bar{p}$a on injection of lactic acid and the effect of reserpine suggests that catecholamines play a part in the response.

Isoprenaline infusion (2 to 4 μg doses in 1 to 2 min), sufficient to raise the heart rate 40 beats per min, immediately abolishes the P$\bar{p}$a response to lactic acid. We find, like others, that β-adrenoceptor blockade restores some of the pressor response removed by α-adrenoceptor blockade.

Several workers (Barer & McCurnie, 1969; Hyman, Woolverton, Guth & Ichinose, 1971) have demonstrated a pulmonary pressor response to various acids and the common factor is the hydrogen ion. Alpha adrenoceptors are particularly concerned in the response but the part played by β-adrenoceptors has yet to be determined. The consistent delay of 9 to 11 s in the appearance of the response remains unexplained

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Hypoglycaemic action of L-DOPA in nialamide treated mice

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5-Hydroxytryptamine, dopamine and their precursors have various effects on blood glucose depending upon the route of injection and whether or not the animals have been fasted or fed. The hyperglycaemic action of L-DOPA and dopamine is well documented (Hakansson, Lundquist & Rerup, 1967). 5-Hydroxytryptophan (5-HTP) injected intravenously (i.v.) or intracerebroventricularly (i.c.v.) and 5-HT injected i.c.v. but not i.v. have been reported to produce hypoglycaemia in mice pretreated with monoamine oxidase inhibitors (MAOI) (Darwish & Furman, 1974). In fed mice pretreated with a monoamine oxidase inhibitor (nialamide 80 mg/kg 20 h and 2 h before blood sampling) both L-DOPA and dopamine produced a dose-dependent elevation in plasma glucose confirming the results of Hakannson *et al.* (1967). However, in fasted, nialamide-treated mice L-DOPA, but not dopamine produced a dose-dependent hypoglycaemic response at 1 h and 2 h after i.v. injection. This response could only be obtained in mice treated with nialamide. DOPA (10 μg) or dopamine (10 μg) each produced hypoglycaemia when injected intracerebroventricularly. This suggested a possible central site of action for L-DOPA. The hypoglycaemic response to L-DOPA was prevented by the dopamine receptor blocking drug haloperidol (0.2 mg/kg s.c. 30 min before injecting L-DOPA). Haloperidol itself had no effect on plasma glucose and did not prevent the hypoglycaemic response to 5-HTP (4 mg/kg i.v.). Cyproheptadine (0.1 mg/kg) or methysergide (0.1 mg/kg) known to block the hypoglycaemic action of 5-HTP (Furman, 1974) each injected 30 min prior to L-DOPA also blocked the response to L-DOPA (20 mg/kg).

Parachlorophenylalanine (PCPA) (300 mg/kg daily for three days p.o.) prevented the hypoglycaemic response to L-DOPA (20 mg/kg or 80 mg/kg) PCPA itself produced an increase in the plasma glucose concentration. Prevention of the response by the 5-HT receptor blocking drugs methysergide and cyproheptadine and by the 5-HT synthesis inhibitor PCPA suggested that the response to L-DOPA might be mediated by 5-HT. However, it was found that PCPA pretreatment also prevented the hypoglycaemic response to 5-HTP (4 mg/kg i.v.) or to 5-HT (10 μg by intracerebroventricular injection). The mechanism of the hypoglycaemic response to L-DOPA in MAOI pretreated mice remains to be determined as does the role of 5-HT in the production of the response.

S.A.E.D. is supported by a W.H.O. Fellowship.

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The relation between the plasma concentration of edrophonium, inhibition of erythrocyte acetylcholinesterase, and the facilitation of neuromuscular function in the rat.

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Although both the biological effects and the pharmacokinetics of edrophonium have been investigated (Randall, 1950; Back & Calvey, 1972a, 1974) little is known of the correlation between them. We have therefore studied the relation between the plasma concentration and the pharmacological activity of the short-acting quaternary amine.

Male Wistar rats (body weight: 250-350 g) were anaesthetized with urethane (1.4 g/kg, i.p.). The contraction of the tibialis anterior muscle evoked by supra-maximal sciatic nerve stimulation (0.33 Hz, 0.5 ms) was measured from both hind limbs. [^{14}C]-edrophonium (dose : 4 or 10 μ mol/kg) was rapidly administered via the jugular vein. Blood samples were collected at intervals for up to 3 hours. Part of the sample was analysed for acetylcholinesterase inhibition by radiometric assay (Potter, 1967; Smith, 1974). (This method abolishes the effects of dilution and

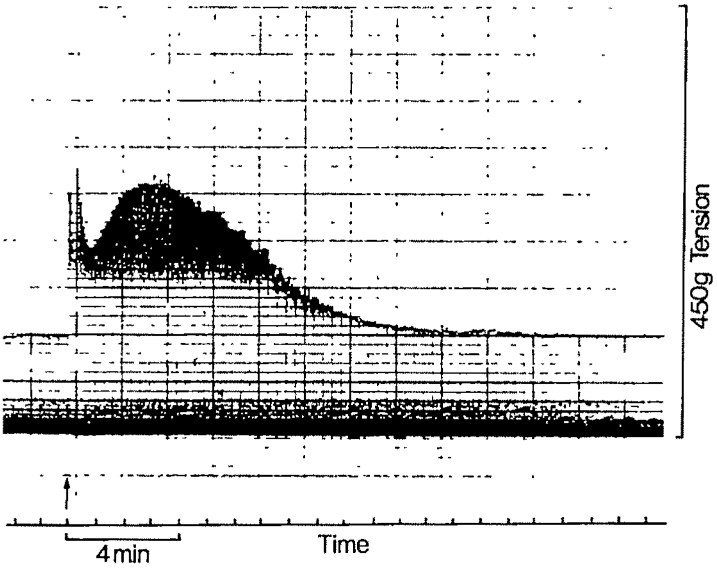

Figure 1. Potentiation by edrophonium (4 μ mol/kg) of the twitch tension of the tibialis anterior muscle evoked by supramaximal sciatic nerve stimulation (0.33 Hz, 0.5 ms).

minimizes the influence of high substrate concentration on reversible cholinesterase inhibitors.) The remainder of the sample was centrifuged and plasma was analysed for edrophonium and its metabolite by liquid scintillation counting after prior chromatographic separation (Back & Calvey, 1972b).

After intravenous injection of edrophonium, acetylcholinesterase inhibition decreased during an experiment from 100% to 69%. In these conditions, the percentage inhibition of acetylcholinesterase was a function of the concentration of the drug in plasma.

Comparable results were obtained *in vitro*. When edrophonium was added to rat blood, a sigmoid relationship was obtained between the logarithm of drug concentration and acetylcholinesterase inhibition. The sigmoid curve was linear between 20% and 80% enzyme inhibition. In addition, there was a statistically significant correlation between acetylcholinesterase inhibition *in vivo* and *in vitro* ($r = 0.99$, d.f. $= 89$, slope $= 1.01$).

Potentiation of the tibialis twitch tension by edrophonium was biphasic (see Figure). This pharmacological effect of edrophonium was not initially correlated with either plasma concentration or acetylcholinesterase inhibition. It is possible that measurement of the tissue levels of edrophonium or a pharmacokinetic prediction of this parameter is correlated with neuromuscular function.

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Presynaptic action of 5-hydroxytryptamine in the myenteric plexus of the guinea-pig ileum

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5-Hydroxytryptamine (5-HT) is known to inhibit the peristaltic reflex when applied to the serosal aspect of the isolated guinea-pig ileum (Kosterlitz & Robinson, 1957) by an action which has been noted to be similar to that of the ganglion-blocking drugs (Kosterlitz & Lees, 1964).

Individual ganglia of the myenteric plexus of the guinea-pig ileum were immobilized by careful pinning and bathed in an organ bath in the manner previously described (Nishi & North, 1973a). Neurones were clearly observed by means of differential interference contrast microscopy (magnification x500) and were impaled with glass micro-electrodes filled with a solution of 3M KCl. Intracellular recordings were obtained from single cells for periods as long as 8 hours. Excitory post-synaptic potentials (e.p.s.p.) were elicited by focal stimulation of the ganglion within a distance of 100 μm from the impaled cell, using a low resistance micro-electrode filled with Krebs solution. A micro-electrode filled with 2M acetylcholine (ACh) chloride solution was manipulated under visual control on to the surface of the impaled neurone. After appropriate adjustment of the position of the micro-electrode tip, iontophoretic currents (retaining current 5-20 nA; ejecting current 10-100 nA, 10-25 ms duration) produced a depolarization of the impaled cell (ACh potential) with a rise time, amplitude and total duration closely similar to the e.p.s.p. in the same cell.

Changes in membrane potential imposed by passing hyperpolarizing or depolarizing currents through the recording micro-electrode affected the e.p.s.p. and the ACh potential in a proportionate manner; the equilibrium potential for the action of the synaptic transmitter was close to that for the iontophoretically applied ACh. Hexamethonium bromide (10-100 μM) reversibly abolished the e.p.s.p. and the ACh potential with a similar time course of action in the two cases.

In confirmation of an earlier report (Nishi & North, 1973b), noradrenaline (1-10 μM) reversibly abolished the e.p.s.p. in all cells to which it was applied. Noradrenaline caused no consistent change in postsynaptic membrane potential or resistance, or in the amplitude of the ACh potential. 5-HT (25 nM – 1 μM) reversibly abolished the e.p.s.p. in most cells, without affecting the amplitude of the ACh potential or changing the postsynaptic membrane potential or resistance. The depression of the e.p.s.p. amplitude produced by 5-HT (200 nM) was rapidly reversed by washing out and could be repeated every 4 min; larger doses of 5-HT caused tachyphylaxis. Methysergide (1.7μ M), which itself slightly depressed the e.p.s.p. amplitude, prevented the action of 5-HT. In a few cells, 5-HT (up to 10 μM) did not change the e.p.s.p. amplitude although noradrenaline had its usual effect.

These findings indicate that 5-HT reduces the release of ACh from most presynaptic terminals within the myenteric plexus. As the only excitatory pathway within the plexus appears to be cholinergic, such as action would inhibit the peristaltic reflex.

Supported by a grant from the U.S. National Institute on Drug Abuse (DA 00662) to H.W. Kosterlitz. G.H. is an I.C.I. Post-doctoral Research Fellow.

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Effect of rubidium on responses of rabbit vas deferens to transmural stimulation

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Transmitter release is known to be very sensitive to changes in the duration of the action potential in nerve terminals (Katz & Miledi, 1967). In the present study, we have examined the influence of rubidium on adrenergic transmission in rabbit vas deferens as rubidium increased the duration of the action potential in squid giant axon (Baker, Hodgkin & Shaw, 1962).

New Zealand rabbits were killed by air embolism. Their vasa deferentia were removed and mounted longitudinally in 5 ml organ baths containing modified Krebs solution at 37° C. The medium contained 3 mM KCl and was equilibrated with 95% O_2/5% CO_2. Contractions were recorded isometrically with strain gauges. Tissues were stimulated transmurally with biphasic pulses of 1 msec duration, supramaximal voltage and 0.5-16 Hz for 60 s every 4 minutes. Transmural stimulation resulted in a rapid twitch response followed by a maintained contraction. This secondary, maintained response resulted from noradrenaline release as it was abolished by phentolamine and guanethidine. In subsequent experiments, the magnitude of the secondary response was determined.

Addition of 2 mM RbCl caused a markedly potentiated response to transmural stimulation at 2-14 Hz both in the presence and absence of 3 mM KCl in the medium. This effect was not due to an impairment of re-uptake of released noradrenaline as the 30 min uptake of [^{3}H](±)-metaraminol was not altered by 2 mM RbCl (with 3 mM KCl, uptake was 175.7 ± 11.2 pmol/g, with 5 mM KCl, 164.8 ± 17.1 pmol/g; with 3 mM KCl and 2 mM RbCl, 179.9 ± 12.8 pmol/g; mean ± s.e. of 10 observations). The responses of vasa deferentia to exogenous (−)-noradrenaline were not altered by 2 mM RbCl thus excluding an effect at receptor level or at subsequent steps in excitation-contraction coupling.

In order to determine the effect of RbCl on transmitter release, tissues were exposed to 10^{-7}M [^{3}H](±)-metaraminol for 60 minutes. After 35 min in an amine-free medium, subsequent transmural stimulation at 5 Hz for 2 min resulted in the release of [^{3}H](±)-metaraminol and this release was very significantly increased by addition of 2 mM RbCl to the medium.

These studies suggest that rubidium may potentiate adrenergic transmission by increasing the release of noradrenaline as has also been reported to occur with caesium (Johns & Paton, 1974).

Supported by a grant from the Medical Research Council of Canada (MT2472).

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Effects of prostaglandins in calves

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The protective effect of sodium meclofenamate against bovine anaphylaxis (Aitken & Sanford, 1972) indicated that prostaglandins might be important mediators in this species. Indomethacin also prevents the systemic hypotension, pulmonary hypertension and apnoea characteristic of anaphylaxis and inhibits Schultz-Dale contractions of isolated bovine pulmonary artery and bronchiole (Aitken & Sanford, unpublished observation).

However, Lewis & Eyre (1972) found that bovine bronchial ring failed to respond to $PGF_2\alpha$ and was relaxed by PGE_1. Effects of other prostaglandins on this tissue have not been

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described. Effects of $PGF_{2\alpha}$, $PGF_{1\alpha}$, PGE_1 and PGE_2 on the cardiovascular and respiratory systems of anaesthetized calves and on isolated pulmonary tissues have been examined in order to clarify the role of prostaglandins in bovine anaphylaxis.

Calves aged 3-4 months, anaesthetized with pentobarbitone, received prostaglandins intravenously. $PGF_{2\alpha}$ (10-60 μg/kg) increased systemic arterial B.P. by 20-85%, pulmonary arterial pressure by 20-200% and heart rate by 3-23%. Respiratory minute volume was reduced by 13-90%. $PGF_{1\alpha}$ (35-140 μg/kg) produced similar increases in systemic pressure, but had no effect on respiration. PGE_1 (1-8 μg/kg) and PGE_2 (2-8 μg/kg) reduced systemic arterial pressure (17-44%), pulmonary arterial pressure (12-37%) and heart rate (5-23%). Respiratory minute volume was reduced by PGE_1 (7-23%) and PGE_2 (26-42%). Tachyphylaxis occurred to $PGF_{2\alpha}$, $PGF_{1\alpha}$, and PGE_2.

Segments of bronchiole and spiral strips of pulmonary artery and vein were suspended in Krebs-Henseleit solution gassed with 95% O_2 and 5% CO_2 at 37°C in organ baths of 80 ml capacity. Contractions were recorded isotonically. Drugs remained in contact with artery and vein for 5 min and with bronchiole for 15 minutes.

PGF_2 contracted bronchiole (0.2-0.3 μg/ml threshold dose), pulmonary artery (0.3 μg/ml) and vein (0.1 μg/ml). All three tissues showed tachyphylaxis. $PGF_{1\alpha}$ (0.5-2 μg/ml) contracted pulmonary artery but concentrations up to 20 μg/ml had no effect on bronchiole. PGE_1 relaxed bronchiole (6-12 μg/ml) and pulmonary artery (0.5-2 μg/ml) but contracted pulmonary vein (1 μg/ml). PGE_2 had variable effects on bronchiole causing contraction (2-5 μg/ml) or, at higher concentrations (6-12 μg/ml), relaxation of muscle contracted by acetylcholine. Pulmonary artery was contracted by PGE_2 (0.4-2 μg/ml), but, when submaximally contracted by serotonin, PGE_2 (5-10 μg/ml) caused relaxation. PGE_2 (1 μg/ml) contracted vein.

Unlike Lewis & Eyre (1972), we found that $PGF_{2\alpha}$ contracted bovine bronchiole as did PGE_2. Human bronchial muscle is contracted by $PGF_{2\alpha}$ but relaxed by PGE_2 (Sweatman & Collier, 1968). Pulmonary hypertensive effects of PGE_1 and PGE_2 have been described in calves (Lewis & Eyre, 1972). The differences in our findings *in vivo* might be related to differences in depths of anaesthesia and smooth muscle tone.

Prostaglandins were generously supplied by Upjohn Ltd., Crawley.

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Teratogenic effects of primidone in mice

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Clinical studies have indicated that there is an increased incidence of congenital malformations, particularly cleft palate, in the infants of mothers exposed to anticonvulsant drugs during pregnancy (Speidel & Meadow, 1972). Since the majority of epileptics in these studies were treated with more than one drug it is not possible to determine exactly which anticonvulsant drugs may possess this teratogenic potential. Of the major anticonvulsants, phenytoin and phenobarbitone have both been implicated as teratogens in animals (Massey, 1969; McColl, Robinson & Globus, 1967), but we are not aware of any reports on the teratogenic effects of primidone in animals.

Primidone was administered to mice derived from the I.C.I. pathogen free strain, either in the diet or by gastric intubation for varying periods from days 6-16 of pregnancy which covers the period of embryogenesis and palatal closure in the mouse. Doses of primidone of 500, 1250, 2000 and 2500 mg/kg administered in the diet on days 6-16 of pregnancy established that primidone was teratogenic; the incidence of cleft palate increased with dose from a control incidence of 0.3% to 29.4% at the highest dose level.

As it has been suggested that low blood folate levels following anticonvulsant therapy might be related to the tetratogenic effects (Elshove, 1969), folic acid (25 mg/kg orally) was administered with primidone (1250 mg/kg). Although folic acid itself was not teratogenic, a significant ($P = 0.0004$) increase in cleft palate was seen with combined treatment compared with primidone alone. However, when folinic acid (3.75 mg/kg s.c.) was administered with primidone (1250 mg/kg) there was a significant decrease in the incidence of cleft palate ($P = 0.03$).

The doses of primidone used in the dietary experiments were far in excess of those used clinically, so much lower doses (100, 150, 250 mg/kg) were given by gastric intubation. All three doses of primidone produced a similar incidence of cleft palate (9-10%) in 50-60% of the litters.

Blood levels of primidone after 100 mg/kg by gastric intubation were measured in pregnant (day 14) mice. A peak level of 42.7 ± 2.8 μg/ml was attained after 30 min and it was virtually cleared from the blood by eight hours. Thus, although the dose of primidone which was teratogenic in mice was about 10 times the therapeutic dose for humans the blood levels obtained were only three to four times higher than those commonly found in patients on primidone (Kutt, 1974).

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A new approach to the evaluation of the safety of flavouring esters

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Experiments have been performed which are designed to determine whether it is possible to reduce the amount of animal experimentation that is needed to assess the safety of flavouring materials. Many of the flavours are esters which are readily hydrolysed *in vivo* to their component acids and alcohols. It is postulated that if the toxicity of each component is known then it might be possible to extrapolate the results to other esters of a particular series, with a consequent economy in the number of animals used and in the experimental time involved.

A series of allyl esters with a straight, branched or a cyclic chain has been investigated. This series was chosen because many pure allyl esters are available and because allyl alcohol produces an unusual effect on the liver—namely periportal necrosis. Allyl alcohol, acetate, propionate, hexanoate, isobutyrate, isovalerate and 2-ethyl hexoate were administered by daily oral intubation in equimolar doses (based on doses of 5, 25 or 60 mg kg^{-1} day^{-1} of the alcohol) to groups of ten male rats. After 21 days the animals were killed and comparisons of the hepatic effects were made. The lesions were classified into three types according to severity; namely periportal cell enlargement, followed by necrosis, and subsequent fibrosis with bile duct hyperplasia.

The severities of the hepatic effects from the straight chain esters were similar to those produced by allyl alcohol and were more marked than those from the branched chain ones. In terms of the number of animals affected, there was a positive relationship between the dose of ester administered and the degree of periportal damage. The animals given the high dose of the straight chain esters exhibited the most extreme lesions and in many instances the effects were so marked as to obscure any signs of early damage. The results of these *in vivo* experiments are in agreement with the *in vitro* hydrolysis studies. The latter indicate that hydrolysis proceeds about 100 times more slowly with the branched chain than with the straight chain esters.

From the data with this series of model compounds it is suggested that it may be possible to evaluate the toxicity of such a series of esters, if the following information is available: (1) the rate and degree of hydrolysis in the alimentary tract, (2) well established data on the parent alcohols and acids, and (3) results of short-term animal experiments, so that it is possible to correlate the *in vitro* hydrolysis studies with the *in vivo* results.

Cardiotoxicity and the plasma digoxin concentration profile in conscious dogs

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Some oral digoxin preparations of high bioavailability produce relatively high peak plasma concentrations and it has been suggested that this could be associated with an increased incidence of toxicity in man (Harter, Skelly & Steers, 1974). The frequency of nausea after oral digoxin at the time of peak plasma concentration supports this concern (Falch, Teien & Bjerkelund, 1973). However, maximal inotropic and chronotropic effects occur several hours after digoxin administration to man, presumably after tissue-plasma equilibrium has been established (Ganz, Fujimori, Penna, Greiner & Gold, 1957; Shapiro, Narahara & Taubert, 1970). It was therefore decided to examine the relationship between cardiac toxicity and plasma digoxin concentration.

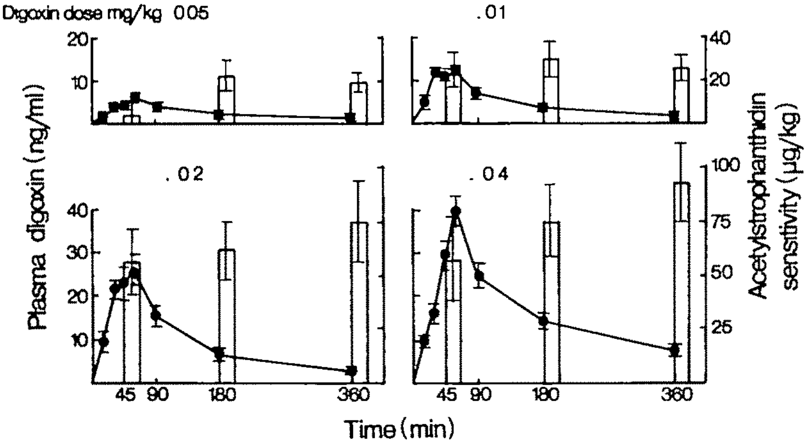

Figure 1 Comparisons of mean plasma digoxin concentration and mean acetylstrophanthidin sensitivity for four oral doses of digoxin administered to eight unanaesthetized dogs. Acetylstrophanthidin sensitivity was calculated as the difference between the amounts of acetylstrophanthidin required to induce cardiotoxicity in the digoxin and control phases.

The plasma digoxin levels are shown by the curves while acetylstrophanthidin sensitivity is represented by the columns. Bars represent standard error about the mean.

The toxic effects on the heart of oral doses of digoxin were determined indirectly at various times after administration to eight conscious dogs by recording the intravenous doses of acetylstrophanthidin necessary to induce cardiotoxic changes. One of the following changes in e.c.g. (Lead II) was accepted as indicative of cardiotoxicity: multifocal ectopics, ectopics representing one in every two beats, ventricular tachycardia or complete heart block. Acetylstrophanthidin (infused at 0.095 mg/min) was used because its rapid elimination from the body permitted separate estimates to be made 45, 180 and 360 min after administration of digoxin. Each dog underwent four studies with 0.05, 0.1, 0.2 and 0.4 mg/kg digoxin given in an alcoholic solution (1.4-14%), allowing at least ten days between each test. Samples of venous blood were taken at intervals for the measurement of plasma digoxin levels by a modified radioimmunoassay technique.

Digoxin reduced the amount of acetylstrophanthidin required to cause toxic changes in the e.c.g. the increase in cardiac sensitivity being dose-dependent. Plasma concentrations of digoxin were maximal 30-60 min after dosing and then declined rapidly (Figure 1). However, sensitivity to acetylstrophanthidin thereafter was maximal between 3 and 6 h after administration of digoxin (Figure 1). At each dose of digoxin there was no correlation between plasma levels and maximal sensitivity to acetylstrophanthidin (Figure 1). These results suggest that there is little or no increased risk of cardiotoxicity during periods of transient increase in plasma digoxin concentration.

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DEMONSTRATIONS

Effect of aporphine alkaloids on central dopamine receptors

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The behavioural syndrome produced in rats after injection of (−)-apomorphine is believed to result from the stimulation of central dopamine receptors. In the present study we have used both *in vivo* and *in vitro* test systems to examine the dopamine-like properties of a series of aporphine alkaloids. Among the compounds tested only (−)-apomorphine and (±)-*N*-n-propylnorapomorphine ((±)-NPA) were effective in stimulating adenylate cyclase activity in homogenates of rat striatum under conditions in which dopamine is known to be effective (Miller, Horn, Iversen & Pinder, 1974). (−)-Apomorphine was slightly more potent than (±)-NPA, but the maximal stimulation seen with both substances was not significantly different. At higher concentrations both compounds caused some inhibition of adenylate cyclase activity. Compounds with the catechol group in a different position, such as (−)-isoapomorphine or (−)-1,2-dihydroxyaporphine, or those lacking one or both of the hydroxyl groups, as in (±)-10-hydroxy-*N*-n-propylnorapomorphine or (−)-aporphine, were found to be inactive in stimulating adenylate cyclase. Several compounds had some ability to inhibit the stimulating effects of 10^{-4} M dopamine on striatal adenylate cyclase. (+)-Bulbocapnine was the most potent compound in this respect, the inhibition was of a competitive nature with a K_i of 1.6×10^{-7} M. 10^{-5} M (+)-Bulbocapnine also inhibited the effects of 10^{-5} M (−)-apomorphine on striatal adenylate cyclase. As (±)-10-hydroxy-*N*-n-propylnorapomorphine has been reported to have some central effects (Neumeyer, Granchelli, Fuxe, Ungerstedt & Corrodi, 1974) we investigated its action following intraventricular injection into rats with unilateral lesions of the nigrostriatal pathway induced with 6-OH dopamine. Injection of 5 μg of (−)-apomorphine produced turning away from the side of the lesion for 40 minutes. However, injection of 25 μg of (±)-10-hydroxy-*N*-n-propylnoraporphine (NPA) was without effect.

Some of the aporphines were also tested for their ability to produce locomotor stimulation in rats with bilateral 6-OH dopamine induced lesions of the nucleus accumbens (see Iversen, Kelly, Miller & Seviour, C.30—this meeting) (±)-NPA was considerably more potent than (−)-apomorphine in producing a stimulation of locomotor activity. Doses of (±)-NPA as low as 0.05 mg/kg (i.p.) produced locomotor stimulation lasting for more than two hours. (−)-Aporphine, (−)-isoapomorphine, (−)-1,2-dihydroxyaporphine and (−)-nuciferine were all without effect. (±)-*N*-n-Propylnorapocodeine, however, produced a long term stimulation of locomotor activity although this compound did not stimulate striatal adenylate cyclase. (−)-Apocodeine has been shown previously to produce stereotypy in rats (Lal, Sourkres, Missala & Belendiuk, 1972). The central activity of (±)-*N*-n-propylnorapocodeine is probably due to its conversion to (±)-NPA in a similar fashion to the conversion of the anti-parkinsonian drug ET495 to its active metabolite S584 (Miller & Iversen, 1974).

R.J.M. is an M.R.C. Scholar. We thank R. Pinder and S. Nahorski for drugs.

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The effect of phosphodiesterase inhibitors on the stimulation of cerebral cyclic AMP formation by biogenic amines *in vitro* and *in vivo*

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In contrast to the situation in several extracerebral tissues, the classical methylxanthine phosphodiesterase inhibitors theophylline and caffeine do not potentiate neurohormone stimulation of cyclic AMP formation in brain slices (Forn & Krishna, 1971; Schultz & Daly, 1973). Recently a number of other compounds have been shown to be more potent inhibitors of phosphodiesterase than theophylline in cell free preparations of brain and we have examined some of these drugs on biogenic amine-stimulated cyclic AMP formation both *in vitro* and *in vivo*.

In vitro experiments were performed on 0.37 mm thick slices of mouse forebrain preincubated for 60 min in Krebs-bicarbonate buffer containing 10 mM glucose. Cyclic AMP was assayed by the method of Brown, Albano, Ekins, Sgherzi & Tampion (1971). Of a number of compounds examined at a concentration of 200 μM, 4-(3-butoxy-4-methoxy)-2-imidazolidinone (Ro 20-1724) and 2-amino-6-methyl-5-oxo-4-*n*-propyl-4,5-dihydro-*s*-triazolo(1,5-a) pyrimidine (ICI 63 197) markedly potentiated the cyclic AMP response to a submaximal concentration (100 μM) of noradrenaline. Theophylline, papaverine and medazepam did not significantly enhance the effect of noradrenaline.

Histamine and dopamine were completely inactive in stimulating cyclic AMP formation in mouse forebrain slices, but in the presence of 200 μM Ro 20-1724 or ICI 63.197, significant (2-3 fold) accumulations of the nucleotide were produced by both of these amines. Prostaglandin E_1 and 5-hydroxytryptamine at concentrations of up to 100 μM did not enhance cyclic AMP formation in the presence or absence of these phosphodiesterase inhibitors.

In this laboratory we have shown that systemically administered biogenic amines increase cerebral cyclic AMP in the neonate chick (Edwards, Nahorski & Rogers, 1974). Using this model an attempt was made to demonstrate inhibition of cerebral phosphodiesterase *in vivo*. Phosphodiesterase inhibitors were injected s.c. or in the case of RO 20-1724 i.v., 20 min before killing. The cerebral hemispheres were removed using a freeze-blowing technique to eliminate post-mortem changes in cerebral cyclic AMP (Nahorski & Rogers, 1973). None of the phosphodiesterase inhibitors alone increased cerebral cyclic AMP *in vivo*. However, the intravenous injection of isoprenaline (5 μmoles/kg) raised brain levels of the nucleotide to 228% of control values in two minutes. In chicks pretreated with RO 1724 (1 mg/kg), isoprenaline increased cerebral cyclic AMP to 342% of levels observed with the phosphodiesterase inhibitor alone. Similarly pretreatment with ICI 63 197 (5 mg/kg) markedly enhanced the response to isoprenaline in that nucleotide levels of 951% above those of controls were observed. Theophylline (50 mg/kg), papaverine (20 mg/kg) and medazepam (20 mg/kg) did not modify the isoprenaline response *in vivo*.

The present results demonstrate that dialkoxyimidazolidinone and triazolopyrimidine phosphodiesterase inhibitors potentiate the increase in cyclic AMP induced by biogenic amines in mouse cerebral slices. Moreover, since these compounds appear to be effective phosphodiesterase inhibitors *in vivo*, they may prove to be valuable tools in assessing the functional role of cyclic nucleotides in the central nervous system.

Financial support from the Wellcome Trust and Stirling Winthrop Ltd. is gratefully acknowledged.

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Plasma tryptophan changes on environmental disturbance and their prevention by propranolol and nicotinic acid

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Plasma tryptophan is largely bound to albumin and the concentration of the small free (ultrafilterable) fraction is considerably influenced by unesterified fatty acids (UFA) which impair the albumin binding. Thus, food deprivation, various lipolytic drugs (Curzon & Knott, 1974) and liver damage (Curzon, Kantamaneni, Winch, Rojas-Bueno, Murray-Lyon & Williams, 1973; Knott & Curzon, 1975) all increase both plasma UFA and free tryptophan and also increase brain tryptophan and 5-hydroxytryptamine (5HT) turnover as indicated by increase of the 5HT metabolite 5-hydroxyindoleacetic acid (5HIAA).

Table 1 Effect of order of removal from cage on plasma unesterified fatty acids and tryptophan concentrations in group caged fasted rats

Order of removal from cage	*UFA mEq/l*		*Tryptophan*	
		Total µg/ml	*free µg/ml*	*% free/l*
1st (5)	0.672 ± 0.106	23.65 ± 2.95	2.75 ± 0.61	11.7 ± 2.39
4th (6) (Killed 6 min later)	0.967 ± 0.161*	17.13 ± 2.64**	3.51 ± 0.46 ns	20.81 ± 3.79**

Results are expressed as means ± one sd. Numbers of determinations are shown in parentheses. Results compared by Student's t-test; * $P < 0.02$; ** $P < 0.01$; ns not significant. Methods were as previously described (Curzon & Knott, 1974).

We now find that when rats (male, Sprague-Dawley, 180-200 g) are housed in groups of 4 and fasted for 24 h their removal from the cages caused UFA and the percent free tryptophan to rise in the plasma of the remaining rats (Table 1). Corticosterone also tended to rise. As total plasma tryptophan fell, absolute changes of free tryptophan were relatively small. The fall of plasma total tryptophan suggests that tryptophan newly freed from albumin binding rapidly leaves the plasma. In contrast to the previous longer term experiments, it did not appear to have entered the brain to any significant extent. Thus, brain tryptophan concentration of first killed fasted rats was 4.12 ± 0.52 µg/g wet wt, (n = 5) which though significantly greater ($P < 0.001$) than that of fed rats (2.19 ± 0.10, n = 3) is essentially the same as that of 4th killed fasted rats (4.05 ± 0.56, n = 6). (Results are ± one s.d.).

The fall in plasma tryptophan was not explicable by increased entry into erythrocytes as erythrocyte tryptophan did not increase. In contrast with tryptophan, plasma tyrosine (which is not bound to albumin) was unaltered. The rapid plasma UFA and tryptophan changes were prevented by intraperitoneal injection of the antilipolytic drugs propranolol (1 mg/kg) or nicotinic acid (50 mg/kg) 1 h before killing. Propranolol did not significantly affect plasma corticosterone while nicotinic acid caused a large increase in fasted rats. This indicates that increases in UFA rather than corticosterone were directly responsible for the tryptophan changes. While nicotinic acid decreased plasma UFA of fasted rats to levels comparable to those of fed animals, propranolol only prevented the increase of UFA due to the environmental disturbance and in agreement with Brodie, Krishna & Hynie (1969) did not alter the underlying elevation of plasma UFA on fasting.

These findings point to propranolol and nicotinic acid as useful pharmacological tools in research on tryptophan metabolism. For example, they could be used to prevent tryptophan changes mediated by the sympathetic–UFA system and thus facilitate study of purely centrally provoked changes of brain 5-HT metabolism.

The M.R.C. are thanked for financial support.

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Histochemical demonstration of an additional form of rat brain MAO

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Monoamine oxidase (MAO) is known to exist in a number of forms, and in the rat brain two types (A & B) have been described by Johnston (1968). Type A-MAO deaminates 5-HT and tyramine and is highly sensitive to inhibition by clorgyline, while the B form deaminates tyramine but not 5-HT and is relatively insensitive to clorgyline. These different forms may well be of considerable importance in the metabolism of monoamines in mammalian brain, and may well have differing distributions (Collins, Sandler, Williams & Youdim, 1970; Goridis & Neff, 1971).

A histochemical method has been developed which permits the demonstration of MAO in rat brain, using both 5-HT and tyramine as substrates. Using clorgyline as an inhibitor A- & B-MAO have been separately demonstrated. Both types are broadly distributed in the rat brain, with a basically similar distribution. Both are present in high amounts in areas known to be rich in monoamines.

A third type of MAO which readily utilizes 5-HT as substrate but is relatively clorgyline insensitive has also been demonstrated. It is predominantly circumventricular in distribution. It is suggested that this form, differing from A- & B-MAO not only in substrate and inhibitor characteristics, but also in distribution should be designated C-MAO. Its distribution suggests a potentially important role for this form of MAO in regulating the movement of biogenic amines between the CSF and the brain.

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Some studies on the purification of monoamine oxidase by affinity chromatography

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Partially purified preparations of monoamine oxidase (MAO) have been prepared by a number of workers (Youdim & Sourkes, 1966; Tipton, 1968, Oreland, 1971) using conventional purification procedures which utilize differences in the chemico-physical properties between MAO and other proteins. Over the past few years affinity chromatographic techniques have been developed using selective adsorbents which have biological affinity for a particular protein and this technique has been utilized to purify enzymes (Cautrecasas, Wilchek & Anfinsen, 1968; Wilchek & Gorecki, 1969).

In the present experiments a number of inhibitors of MAO were used as ligands and were attached to sepharose columns in an attempt to purify MAO by a single step experimental procedure.

An organomercurial-sepharose column was prepared by utilizing p-chloromercuribenzoate as the ligand. p-Chloromercuribenzoate was added to aminohexane sepharose suspended in 40% dimethyl formamide. 1-Ethyl-3-(3-dimethylamino propyl) carbodiimide was added, the pH maintained at 4.8 and the mixture allowed to react for 18 h at room temperature. The gel was then washed with 0.1 M $NaHCO_3$ followed by distilled water and was then equilibrated with 1 mM phosphate buffer pH 7.8. Sonicated soluble rat liver MAO was then applied to the gel and washed through with buffer. The MAO enzyme was eluted in the unbound fraction and was 5.6 times purer than the starting material with a 100% recovery of the enzyme. The bound protein was eluted from the organomercurial-sepharose column with 50 mM cysteine in 0.2 M phosphate buffer at pH 7.4 containing 0.01 M EDTA.

A tranylcypramine-sepharose column was prepared by attaching tranylcypramine to succinyl aminohexane sepharose. Soluble MAO applied to this column was not bound nor was there an increase in the specific activity of the eluate from the column.

A sepharose column was also prepared by attaching 1-meta-aminophenyl-2-cyclo-propyl-aminoethanol (AB-15), to succinyl amino hexane-sepharose. Soluble MAO was applied to this column and 10% of the enzyme together with 15% of the protein was bound to the column. Elution of the column with 0.2 M phosphate buffer, pH 7.6, recovered only 1% of the enzyme, but this fraction had a 40 fold purification over the original material.

The results suggest that affinity chromatographic techniques may provide a suitable procedure for preliminary purification of MAO.

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Behavioural and biochemical evidence for cerebral dopamine receptor blockade by metoclopramide in rodents

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Metoclopramide, a widely used anti-emetic agent, causes dyskinesias in a small percentage of patients (Borenstein & Bles, 1965; Casteels-van Daele, Jaeken, van der Schueren, Zimmerman & van der Bon, 1970); like other neuroleptic agents which cause similar dyskinesias, it may block cerebral dopamine receptors (Costall & Naylor, 1973; Janssen, Niemegeers, Schellekens & Lenaerts, 1967). These behavioural and biochemical studies described here indicate that metoclopramide is a cerebral dopamine receptor antagonist.

Metoclopramide administered i.p. 30 min before apomorphine (2 mg/kg) produced a dose-dependent inhibition of stereotopy (ED_{50} 1.5 mg/kg). The reversal of reserpine-induced suppression of locomotor activity by apomorphine (2 mg/kg) was significantly decreased by prior administration of metoclopramide (ED_{50} 17 mg/kg). Metoclopramide also antagonized the effect of apomorphine or amphetamine in producing turning behaviour in mice with unilateral lesions of the nigrostriatal pathway (ED_{50} 5.0 and 4.0 mg/kg respectively). Metoclopramide resembled pimozide in all these respects and appeared to be a relatively potent antagonist of striatal dopamine receptors in these behavioural models (Dolphin, Jenner, Marsden, Pycock & Tarsy, 1975).

Pretreatment of male mice (20-30 g) with metoclopramide (50 mg/kg i.p.) caused a time-dependent increase in homovanillic acid (HVA) in whole brain, which was maximal (740%) 1½ h after injection and lasted some 5 hours. Metoclopramide (50 mg/kg) increased HVA concentrations to the same extent in the corpus striatum (450%) and the mesolimbic area (590%) (a slice containing corpora amygdala, nucleus accumbens and olfactory tubercle). On the other hand no change in whole brain concentrations of dopamine, noradrenaline (NA), 4-hydroxy-3-methoxyphenylglycolsulphate (MOPEG-SO4), 5-hydroxytryptamine (5HT), or 5-hydroxyindoleacetic acid were observed after metoclopramide treatment. The biochemical results suggest that metoclopramide blocks cerebral dopamine receptors in both striatum and mesolimbic areas, thereby increasing dopamine turnover. On the other hand the noradrenaline and 5-hydroxytryptamine systems do not seem to be influenced. (Since this work was completed Ahtee (1975) has also independently reported that metoclopramide causes a rise in whole brain HVA.)

It remains to be discovered why metoclopramide given to parkinsonian patients does not increase the severity of the parkinsonian syndrome, and does not affect the intensity of L-DOPA induced dyskinesias (Tarsy, Parkes & Marsden, 1975). It is also surprising that metoclopramide has little, if any, antipsychotic activity (Borenstein & Bles, 1965) in view of the current suggestion that this property is associated with the capacity to block cerebral dopamine receptors particularly those in the mesolimbic area (Van Rossum, Janssen, Boissier, Julou, Loew, Moller Nielsen, Munkrad, Randrup, Stille & Tedeschi, 1970, Costall & Naylor, 1973).

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Measurement of metabolism of resting and exercising human skeletal muscle *in situ.*

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Forearm blood flow is measured by the venous occlusion plethysmographic method and the flow through the muscle determined from the measured total forearm flow (Cooper, Edholm & Mottram, 1955). By catheterization of deep forearm veins from the antecubital fossa it is possible to obtain samples that are predominantly muscle effluent blood (Coles, Cooper, Mottram & Occleshaw, 1958). The simultaneous sampling of arterial blood enables arterio-venous differences across the muscles to be determined of various substances of metabolic importance. A.-V. differences multiplied by blood flow rates give the metabolic turn-over rates for these substances. Use of an isometric hand-grip ergometer, with hand grips of 5 or 10% of subjects' maximal power, allows the blood flow and sampling techniques to be performed while the muscle is exercising under controlled conditions as well as at rest (Baker & Mottram, 1973).

We are developing these methods to study the actions of substances, both naturally occurring hormones and pharmacological agents, believed to affect the tissue's metabolic activity.

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Δ^1-Tetrahydrocannabinol and adrenergic mechanisms

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Δ^1-Tetrahydrocannabinol (THC) has been shown in the past to possess several cardiovascular actions in both animals and man (Graham & Li, 1973; Davies, Weatherstone, Graham & Griffiths, 1974; Marshall, unpublished observations). The basic pharmacological mechanisms underlying these actions are complex and have not yet been fully eludicated.

Using isotope studies Graham, Lewis & Li (1974a) have shown that Δ^1THC in doses ranging from 1.1×10^{-7} M to 1.4×10^{-5} M inhibited the uptake of ^{3}H-l-noradrenaline (NA) into the isolated, perfused rat heart, the ID_{50} value being 1.4×10^{-5} M. These workers (1974b) also found that in rat vasa deferentia, the amount of ^{3}H-l-NA released spontaneously or as a result of transmural electrical stimulation was reduced in the presence of Δ^1-THC in similar concentrations to the above. The reduction was dose-dependent. Rat vasa pre-incubated with ^{3}H-Δ^1-THC released significantly more tritium into the bath fluid in a given period of time when stimulated than not stimulated. Pretreatment of rats with 6-hydroxydopamine (6-OHDA) in a dose which destroys adrenergic nerves (Furness, Campbell, Gillard, Malmfors, Cobb & Burnstock, 1970) abolished this difference, suggesting that the release of Δ^1-THC from the stimulated vas deferens came, at least in part, from the adrenergic nerve. Further evidence that Δ^1-THC is taken up into the adrenergic neurone has recently been obtained by Egan, Graham & Lewis (unpublished observations). These workers have shown that the isolated rat vas deferens incubated for varying times with ^{3}H-Δ^1-THC retained significantly more tritium prior to treatment of the animals with 6-OHDA than afterwards.

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Ultracryotomy for high resolution drug-localization studies

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The usefulness of high resolution autoradiographic drug-localization studies is severely limited by the ability of most drugs to diffuse from the receptor sites during lengthy preparative procedures. To overcome this disadvantage, work is in progress to obtain ultra-thin (ca. 100 nm) sections of fresh, unprotected, unfixed, unstained tissue which is rapidly frozen using a variety of quenching agents.

Evidence is presented that sections are cut (and not formed by fracturing) using glass or diamond knives at temperatures as low as −125° C, using a Huxley Mark II ultramicrotome with a cryo-attachment (Cambridge Instruments). Sections are picked up on carbon coated grids and transferred to the coating unit where they are allowed to warm up to room temperature under vacuum. They may or may not be top-coated with a layer of carbon before examination in a Philips 300 electron microscope.

Electron micrographs of liver sections prepared as above are displayed, together with micrographs illustrating artifacts arising from overlaying sections by carbon evaporation, allowing sections to warm up in air, and ice-crystal formation.

A new technique utilizing contact adhesive for applying photographic emulsion to frozen-cut sections is reported.

This work is supported by a grant from the Medical Research Council.

Metyrapone inhibits prostaglandin synthesis and release from the pregnant rat uterus *in vitro*

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Metyrapone, an inhibitor of corticosteroid biosynthesis (Chart & Sheppard, 1959), causes foetal adrenal hypertrophy (Dupouy, 1972) and prolongs pregnancy in the rat (Parvez, Parvez & Roffi, 1972). As the foetuses exert some form of control over prostaglandin (PG) release from the pregnant rat uterus *in vitro* (Parnham, Sneddon & Williams, 1975), the effect of metyrapone on this uterine PG release has been investigated.

Two experimental approaches were used. First, pregnant rats received daily injections of either metyrapone (150 mg kg^{-1}, s.c. or i.p.) or vehicle (0.33 M (+) tartaric acid, 0.75 ml kg^{-1}, s.c. or i.p.) on days 20 and 21 of pregnancy. The animals were then killed on day 22 and the PG release from single uterine horns determined by the method of Vane & Williams (1973). Secondly, PG synthetase activity in day 22 pregnant uterine homogenates was determined by incubation for 10 min with ^{14}C-arachidonic acid and measuring its conversion to PGE_2 and $PGF_{2\alpha}$ which were separated on silicic acid mini-columns. Incubations were carried out in the presence of metyrapone (0.005-0.05 mM) against boiled blanks and buffer controls.

Metyrapone significantly reduced uterine PG release *in vitro* from 253 ± 35.5 (s.e. mean) ng g^{-1} h^{-1} in control uteri to 174 ± 21.2 ng g^{-1} h^{-1} in metyrapone-treated uteri ($P < 0.05$). The results of Parvez *et al.* (1972) showing prolongation of pregnancy by metyrapone (150 ng kg^{-1}) were also confirmed in a group of 13 rats. Metyrapone showed a dose-dependent inhibition of PGE_2 synthesis *in vitro* as determined by the conversion of ^{14}C-arachidonic acid to PGs. $PGF_{2\alpha}$ synthesis showed an apparent stimulation with metyrapone, probably due to redirection of synthesis from PGE_2. Preliminary experiments with indomethacin have confirmed that this inhibitor blocks the synthesis of both PGE_2 and $PGF_{2\alpha}$.

These results suggest that metyrapone is a selective inhibitor of uterine PGE_2 synthesis and this may account for the prolongation of pregnancy by metyrapone in the rat.

M.J.P. is a Medical Research Council Scholar.

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Action of spasmogenic substances on Ca^{2+} movements of rat uterine smooth muscle

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These experiments were performed on uteri obtained from virgin Wistar rats of 180-200 g weight, pretreated the day before the experiment with 0.5 mg/kg of diethylstilboestrol diproprionate. Myometrium strips were prepared by excising the mucous and submucous layers.

The role of extracellular Ca^{2+} in myometrial contractility was examined on strips mounted isotonically. In at least five experiments angiotensin II (At II) 2.5×10^{-6} M, serotonin (5-HT) 2.5×10^{-5} M and carbachol (CCh) 2.5×10^{-5} M, produce a maximum contraction in a normal Ringer solution. In each experiment the contraction produced was less than 10% of the control as soon as 3 min after exposure of the strips to a solution without Ca^{2+}. The contractile response was restored to control levels less than 3 min after readmitting Ca^{2+} 1.5 mM to the bath. These results suggest that: (1) activation of contraction is due to an increase in membrane permeability to Ca^{2+}, or (2) extracellular Ca^{2+} is necessary to maintain intracellular Ca^{2+} stores at a normal level. In order to examine these possibilities, we have studied the action of the spasmogenic substances on ^{45}Ca uptake and efflux. ^{45}Ca uptake was determined with the La^{3+} technique (Mayer, Van Breemen & Casteels, 1972). At II 2.5 x 10^{-6} M, and CCh 2.5 x 10^{-5} M did not produce any significant change in uptake curves. On the other hand, KCl 101 mM increased ^{45}Ca uptake at 5, 10, 20 and 30 min exposure to the radioactive solution as well as the exchangeable Ca^{2+} that increased from the control value of 0.163 ± 0.007 mM/kg to 0.244 ± 0.013 mM/kg (10), $P < 0.02$. 5-HT 2.5 x 10^{-5} M also augmented Ca^{2+} uptake at 5, 10, 20 and 30 min, as well as the exchangeable Ca^{2+} which increased from 0.168 ± 0.009 mM/kg to 0.200 ± 0.008 (14), $P < 0.01$

Efflux curves were performed after previous equilibration with the ^{45}Ca Ringer solution. The ^{45}Ca efflux was obscured by the Ca^{2+} exchange with the binding sites in the extracellular space. In order to dissociate this latter exchange from the efflux from the cellular compartment, we displaced the Ca^{2+} bound to the extracellular and superficial membrane sites by adding 20 mM Ca^{2+} ethylene glycol-bis (β-amino-ethyl ether) N,N′-tetraacetic acid (EGTA) (Van Breemen & Casteels, 1974). Under these conditions AtII 2.5 x 10^{-6} M did not modify the rate of efflux of rat myometrium which varied from 13.7 ± 1.3 to 15.36 ± 0.6 min^{-1} x 10^{-3} (8), $P < 0.20$. On the contrary, efflux was augmented by 5-HT 2.5 x 10^{-5} M from 10.52 ± 0.6 to 16.27 ± 1.5 min^{-1} x 10^{-3} (4), $P < 0.01$; by CCh 2.5 x 10^{-5} M from 11.5 ± 0.9 to 14.25 ± 0.5 min^{-1} x 10^{-3} (4), $P < 0.01$ and by KCl 101 mM from 10.20 ± 0.1 to 13.70 ± 0.1 (4), $P < 0.05$. These increases of efflux may be due to a greater membrane permeability to Ca^{2+} or to the release of Ca^{2+} from intracellular binding sites.

In conclusion, AtII and CCh activate uterine smooth muscle contraction by a displacement of Ca^{2+} from intracellular stores, since Ca^{2+} uptake was not affected. The Ca^{2+} stores were in equilibrium with extracellular Ca^{2+}. On the other hand, KCl and 5-HT act at least in part by increasing the membrane permeability to Ca^{2+}.

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A comparison of some smooth-muscle effects of GABA and of prostaglandin E$_1$

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The effects of GABA on intestinal smooth muscle preparations are variable, one of the most regular being inhibition of the ACh- or nicotine-induced contractions of the guinea-pig ileum (Hobbiger, 1958; Florey & McLennan, 1959). We have found direct excitatory effects from high concentrations of GABA on the guinea-pig uterus or caecal taenia. These effects may perhaps be more closely related to the depolarizing action of GABA on some nerve cells than to its hyperpolarizing action on others. The uterus and taenia also contract in response to PGE$_1$ in much lower concentrations; but the longitudinal muscle of the ileum or colon is less sensitive to either spasmogen.

The concentrations of GABA needed to cause contraction are generally within the range 1-30 mmol/l, but potentiation of other spasmogens has been seen in some experiments on the uterus with 0.15 mmol/l. In some experiments, the time-course of this potentiation has resembled the prolonged 'enhancement' following brief application of PGE$_1$ (Clegg, Hall & Pickles, 1966). At the higher concentrations, the metabolism of GABA might provide a significant amount of energy. However, the GABA-transaminase inhibitor amino-oxyacetic acid (10-45 μg/ml) neither consistently increased nor decreased the uterine responses.

Atropine (10 μg/ml) did not alter the responses of either tissue to GABA, and neither bicuculline nor its methochloride (10-25 μg/ml) clearly inhibited the responses of the uterus. Those to PGE$_1$, and to GABA in a few experiments, were partially inhibited by the prostaglandin-antagonist diphloretin phosphate (DPP, 5-10 μg/ml) but not by 7-oxa-prost-13-ynoic acid (1-10 μg/ml), the latter sometimes being spasmogenic. In these respects, the responses of the uterus both to GABA and to PGE_1 were 'atypical'.

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The Demonstration consists mainly of records illustrating these statements.

We wish to thank Professor D.A. van Dorp for the PGE_1, Professor J. Fried for the 7-oxa-prost-13-ynoic acid, and Professor A.H. Jackson for the preparation of bicuculline methochloride.

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A superfusion system used for loading and washing out ^{45}Ca from frog ventricular strips

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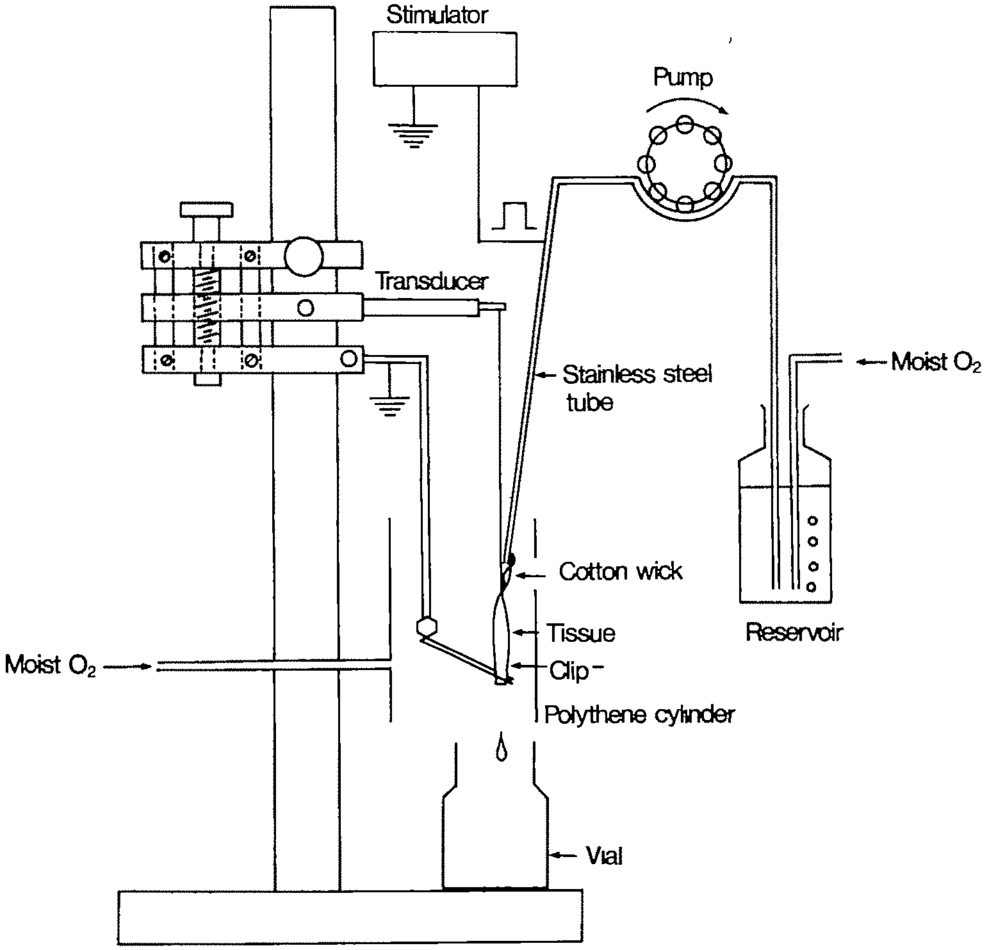

Figure 1 Diagram of superfusion system not drawn to scale. A separate recirculating circuit was used for superfusion with radioactive loading solution.

Techniques available for investigating radioisotope effluxes from isolated muscles include those of sequential dipping and continuous flow. The former have the disadvantage that the tissue is subjected to repeated changes in its environment, and that dipping produces artifacts on any mechanical record. Continuous-flow systems may give rise to large volumes of effluent solution if washing is to be effective.

Our system has the following advantages. It is simple and lends itself to paired studies. The muscle is in a constant environment and its mechanical activity can be recorded. The flow of washing solution is minimized, while effective washing is assured.

The preparation was a strip of 1-1.5 mm diameter, cut from the ventricular wall of *Rana temporaria* and mounted in the apparatus as shown in Figure 1. The lower end of the strip was clipped, while the upper end was attached by thread to an E.E.L. 2.S.T.02 force transducer. The strip was superfused with frog Ringer solution applied via a stainless steel tube and cotton wick, which also served as one stimulating electrode. Stimulation frequencies of 0.25-0.5 Hz were used. Ringer solution drained from the preparation at 0.25-0.5 ml/min into waste (equilibration), recirculating reservoir of ^{45}Ca-labelled Ringer (loading), or collecting vials (wash-out).

The preparation, at room temperature, was in an atmosphere enriched with moist O_2, and proved to be viable for long periods. It was possible to record its mechanical activity during equilibration, loading and wash-out. Superfusing solutions could be changed during wash-out without mechanical artifacts by transferring the solution intake tube quickly into a fresh reservoir.

Sufficient points could be obtained to construct accurate efflux curves, and detect rapid changes in the rate of efflux. With this system, we have shown transient increases in efflux rate with increases of the sodium/calcium concentration ratio in the medium, or addition of 5-10 mM La^{3+}.

An indirect blood pressure measuring system with digital readout

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A system has been designed to provide a fast, accurate indirect measurement of systolic blood pressure in rats immobilized by light ether anaesthesia as recommended by Byrom (1947).

A block diagram of the apparatus is shown. The rats are put for 15 min in a cabinet maintained at 37° C, lightly anaesthetized with ether, placed on a platform, also at 37° C, with a sphygmomanometer cuff and sensor positioned on the tail.

The control unit inflates the cuff to 300 mmHg and then allows it to slowly deflate until the systolic pulse is detected. The cuff pressure is monitored by the transducer and converter and on receiving the arterial pulse via the sensor the systolic blood pressure is displayed digitally. A push button connected to the release valve allows rapid deflation of the cuff. The sequence of operations takes approximately 30 seconds.

The apparatus utilizes standard 74 series logic circuitry and as the endpoint is determined electronically errors due to misreading of the manometer or observer bias are avoided.

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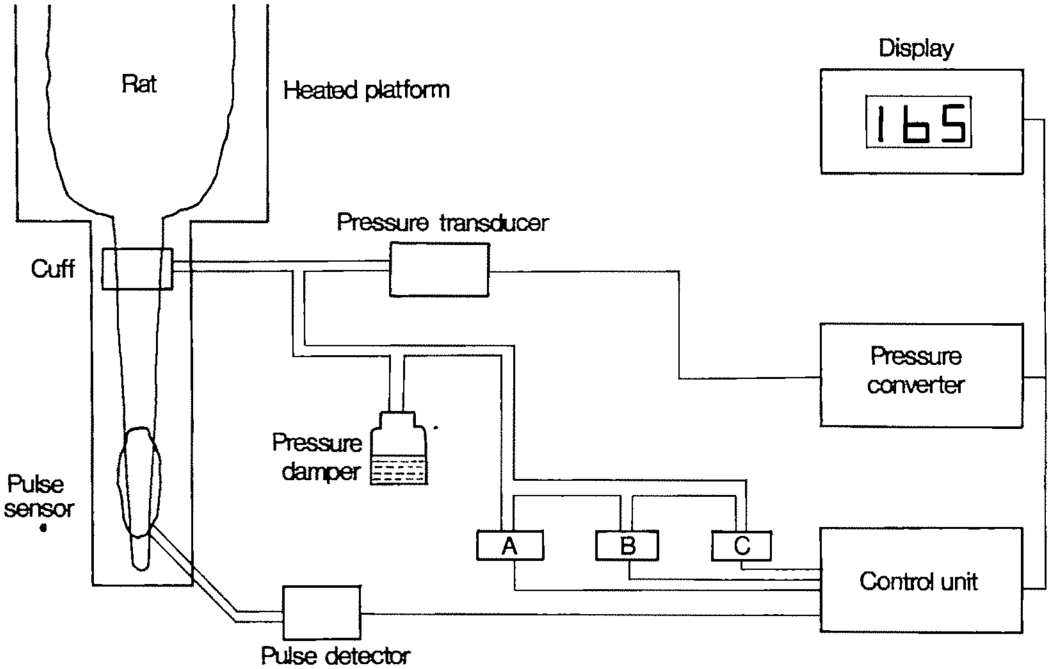

A- Inflation valve B-Slow deflation valve C-Release valve

INSTRUCTIONS TO AUTHORS

Papers will be considered for publication on all aspects of drug action, including chemotherapy. A recent issue of the Journal is a good guide to style.

Manuscripts (two copies) should be sent to Dr H.M. Adam, Department of Pharmacology, University of Edinburgh Medical School, 1 George Square, Edinburgh, EH8 9JZ, from whom copies of the full Instructions to Authors, published in *Br. J. Pharmac,* **50**, 3-23, may also be obtained. Specific points to note are

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Printed in Great Britain by The Whitefriars Press Ltd, London and Tonbridge

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July 1975

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The British Journal of Pharmacology is published monthly by Macmillan Journals Ltd, 4 Little Essex Street, London WC2R 3LF. Telephone: 01-836 6633.

Annual subscription £36.00 ($90 USA and Canada). (Orders must be accompanied by remittance. Prices applicable only to orders received before 31st December 1975.)

Cheques should be made payable to Macmillan Journals Limited: Subscription Department, Brunel Road, Basingstoke, Hampshire RG21 2XS, England.

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Systematic Pharmacology

SP1	Cardiovascular system
SP2	Central nervous system
SP3	Other physiological systems

Pharmacokinetics

PK1	Storage, distribution, fate
PK2	Synthesis, metabolism
PK3	Uptake, release

CP	**Clinical Pharmacology, Experimental Therapy**
CT	**Chemotherapy**
M	**Miscellaneous**

Autopharmacology

AP1	Adrenergic, cholinergic transmission
AP2	Histamine, kinins, prostaglandins, etc
AP3	Hormones

Drug Mechanisms

DM1	Molecular, cellular, biochemical, biophysical
DM2	Receptors, antagonism, potentiation, tolerance

PM	**Pharmacometrics**
PP	**Psychopharmacology**
TX	**Toxicology**

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EFFECTS OF DESIPRAMINE ON NEURONAL RESPONSES TO DOPAMINE, NORADRENALINE, 5-HYDROXYTRYPTAMINE AND ACETYLCHOLINE IN THE CAUDATE NUCLEUS OF THE RAT

P. BEVAN, C.M. BRADSHAW & E. SZABADI
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1 The sensitivity of single neurones to microelectrophoretically applied dopamine, noradrenaline (NA), 5-hydroxytryptamine (5-HT) and acetylcholine (ACh) was investigated in the caudate nucleus of the rat, anaesthetized with halothane. Both excitatory and depressant responses could be observed to each of the agonists. There was a high correlation between the direction of responses to dopamine and noradrenaline, whereas there was no significant correlation between the direction of responses to dopamine and ACh.

2 The effect of desipramine was studied on both excitatory and depressant responses to dopamine, NA and 5-HT, and on excitatory responses to ACh Both potentiation and antagonism of neuronal responses to monoamines and ACh could be observed after a brief application of desipramine.

3 Excitatory responses to glutamate were not affected by desipramine.

4 The observation that responses to dopamine and NA can be potentiated by desipramine in the caudate nucleus suggests that uptake blockade is not a prerequisite for potentiation.

5 It is suggested that the potentiation of neuronal responses to dopamine by desipramine may be responsible for the therapeutic efficacy of desipramine in Parkinson's disease.

Introduction

It is generally believed that the symptomatology of Parkinson's disease is caused by the selective degeneration of the dopamine-containing nigro-striatal pathway, which leads to an impaired balance between dopaminergic and cholinergic inputs in the striate nucleus (Barbeau, 1962; Klawans, 1968) Drugs effective in the treatment of Parkinson's disease are thought to act either by inhibiting the effects of acetylcholine (e g. atropine), or by enhancing the effects of dopamine (e.g. L-DOPA) (Klawans, 1968, Yahr & Duvoisin, 1972).

It has been reported that desipramine, a tricyclic antidepressant drug, is effective in the treatment of Parkinsonism (Laitenen, 1969) The basis for the anti-Parkinsonian efficacy of desipramine, however, is not known. Although the cholinolytic effects of desipramine are well documented (Atkinson & Ladinsky, 1972), it is not likely that this is the sole explanation for the anti-Parkinsonian efficacy of the drug, since the therapeutic effectiveness of desipramine is greatly enhanced by the concurrent administration of another cholinolytic drug (Yahr & Duvoisin, 1972). Another possibility could be that desipramine potentiates the effects of dopamine in the caudate nucleus. Such an effect, however, could not be predicted on the basis of the well known 'uptake blockade hypothesis of potentiation' (Iversen, 1974), since desipramine is almost completely ineffective in blocking the uptake of dopamine in the caudate nucleus (Ross & Renyi, 1967; Horn, Coyle & Snyder, 1971).

We have reported earlier that desipramine has a dual effect on neuronal responses to noradrenaline (NA), 5-hydroxytryptamine (5-HT), and acetylcholine (ACh) in the cerebral cortex: both antagonism and potentiation of the responses can be observed (Bradshaw, Roberts & Szabadi, 1974, Bevan, Bradshaw & Szabadi, 1975a). In the experiments described here we used the technique of microelectrophoresis in order to examine how neuronal responses to dopamine, NA, 5-HT and ACh can be affected by desipramine in the caudate nucleus of the rat.

Some of the results presented here have been communicated to the British Pharmacological Society (Bevan, Bradshaw & Szabadi, 1975b).

Methods

Male albino Wistar rats, weighing between 250 and 300 g were used. Anaesthesia was induced with halothane (3.0%), and maintained with halothane (0.5-1.0%) delivered from a temperature and flow-rate compensated vapouriser (Fluotec Mk 3, Cyprane Ltd). All animals respired spontaneously via a tracheal cannula. ECG and EEG were monitored continuously throughout the experiment. Rectal temperature was maintained between 37° C and 38° C with a heating pad controlled by a thermosensitive rectal probe.

The head of the animal was held rigidly in a stereotaxic frame. A small hole was prepared in the skull (coordinates: A: 8 mm, L: 2.4 mm; König & Klippel, 1963), and a small area of the cortex exposed according to the method of Bradshaw & Szabadi (1972). A six-barrelled micropipette was then introduced into the brain, under microscopic control, and slowly lowered through the cortex into the caudate nucleus.

Six-barrelled glass micropipettes were constructed and filled as described by Bradshaw, Roberts & Szabadi (1973). Two barrels of each micropipette contained 4 M NaCl, one barrel for recording action potentials, the other for use in current balancing. The remaining barrels contained drug solutions. The following drug solutions were used: dopamine hydrochloride (0 2 M, pH 4.0-4.5), noradrenaline bitartrate (0.2 M, pH 3.0-3.5), 5-hydroxytryptamine bimaleate (0.2 M, pH 3.5), acetylcholine chloride (0.2 M, pH 3.6), sodium glutamate (0.05 M, pH adjusted to 8.5 by the addition of 0.1 M NaOH), and desmethylimipramine hydrochloride (0.15 M, pH 7.5).

The techniques for recording action potentials, and for the electrophoretic application of drugs, were as described by Roberts & Straughan (1967). A cumulative record of the total number of action potentials was obtained via a Grass UI-1 unit integrator.

All the neurones studied were spontaneously active. All the drugs were applied by microelectrophoresis. Repeated responses to an agonist were compared before and after a brief application of desipramine. In order to ensure that standard ejecting current pulses gave rise to standard pulses of drug ejection (Bradshaw, Szabadi & Roberts, 1973), the intervals between drug applications were kept constant by means of a sequential timing device (Bevan & Bradshaw, 1973). Cells were excluded from drug interaction studies if the variation in the size of the control responses to an agonist exceeded ± 10% (see Bradshaw *et al.*, 1974).

The magnitude of a response was measured by calculating the difference between the number of spikes generated during the response and the number of spikes generated during an equivalent period when no drug was applied ('total spike number', see Bradshaw *et al.*, 1974).

Results

Responses to monoamines and acetylcholine

The effects of dopamine, NA, 5-HT and ACh were studied on 208 spontaneously active neurones in the caudate nucleus. Three types of responses could be observed: (1) excitation; (2) depression; (3) biphasic responses, consisting of an initial depressant phase followed by an excitatory phase. Table 1 shows the frequency of occurrence of these effects in our material. In eight cells a 'spontaneous reversal' of the response to the monoamine was observed: the cell first responsed with either a clear excitation or a depression to the monoamine; later, however, the same monoamine evoked an opposite response (four cells with dopamine, two cells with NA, two cells with 5-HT). Cells showing 'spontaneous response reversal' were not used for drug interaction studies.

In 48 cells responses to dopamine and to another agonist (NA, 5-HT, ACh) were compared. (In a number of cells responses to dopamine and to two other agonists were tested.) Table 2 shows the correlation between the direction of the response (excitation or depression) to dopamine and the direction of the response to the other agonist. It is apparent that there was a highly significant correlation between the effects of dopamine and NA, whereas there was no significant correlation between the effects of dopamine and 5-HT. Furthermore, there was no significant correlation between responses to dopamine and ACh.

Effect of desipramine on neuronal firing

The direct effect of desipramine on the firing rate was studied on 65 cells. The dose of antidepressant applied was 30-100 nA passed for 30-80 seconds. In six cells (9%) the firing rate was increased during the application of desipramine, whereas in 19 cells (29%) the firing rate was decreased. There was no significant correlation between the dose of desipramine applied and the effect on neuronal firing. The response was always of a temporary nature, and the original base line firing rate recovered within a minute after the application of desipramine had been terminated.

Effect of desipramine on responses to dopamine

Excitatory responses Both potentiation and antagonism of excitatory responses to dopamine could be observed after a brief application (30-100 nA for 30-80 s) of desipramine.

Potentiation of the response was seen in five cells. A response was regarded as potentiated if there was more than 20% increase over the size of the mean control response (Bradshaw *et al.*, 1974). An example of potentiation is shown in Figure 1. Antagonism of the response to dopamine was seen in six cells. A response was regarded as antagonized if there was more than 20% decrease in the size of the response compared to the mean

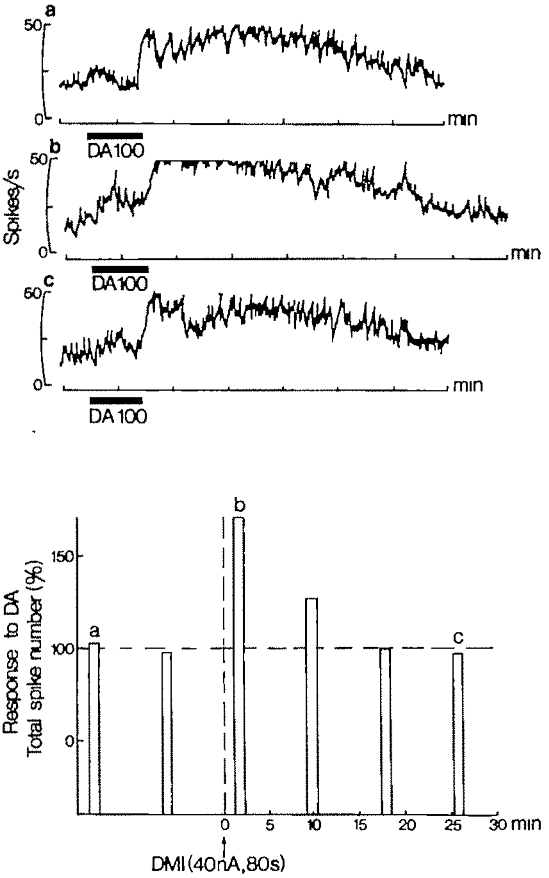

Figure 1 Potentiation of excitatory responses of a single caudate neurone to dopamine (DA) by desipramine (DMI). Top of the figure shows excerpts from the ratemeter recording of the firing rate of the neurone. Horizontal bars indicate applications of dopamine; numbers refer to the intensity of the ejecting current (nA). (a) Control response to dopamine, (b) potentiated response to dopamine 1 min after a brief application of desipramine (40 nA; 80 s); (c) recovery of control response 25 min after the application of desipramine. The graph at the bottom shows the time course of the entire study. The sizes of the responses to dopamine are expressed as a percentage of the mean of the control responses. Each column represents a single response. Letters above the graph indicate responses illustrated in the rate-meter tracings above.

Table 1 Responses of caudate neurones to monoamines and acetylcholine

	Response (number of cells)		
Agonist	*Excitation*	*Depression*	*Biphasic*
Dopamine	33 (53%)	21 (34%)	8 (13%)
Noradrenaline	32 (50%)	26 (41%)	6 (9%)
5-Hydroxytryptamine	32 (64%)	12 (24%)	6 (12%)
Acetylcholine	52 (91%)	4 (7%)	1 (2%)

Table 2 Correlation between the effects of dopamine and of other agonists on caudate neurones

		Direction of responses to the two agonists		*Significance of correlation (χ^2 test)*
*Agonists compared**		*Same*	*Opposite*	
Dopamine, noradrenaline	(33)	91%	9%	$P < 0.001$
Dopamine, 5-hydroxytryptamine	(19)	79%	21%	NS
Dopamine, acetylcholine	(17)	71%	29%	NS

* Figures in parentheses indicate number of cells on which comparisons were made.

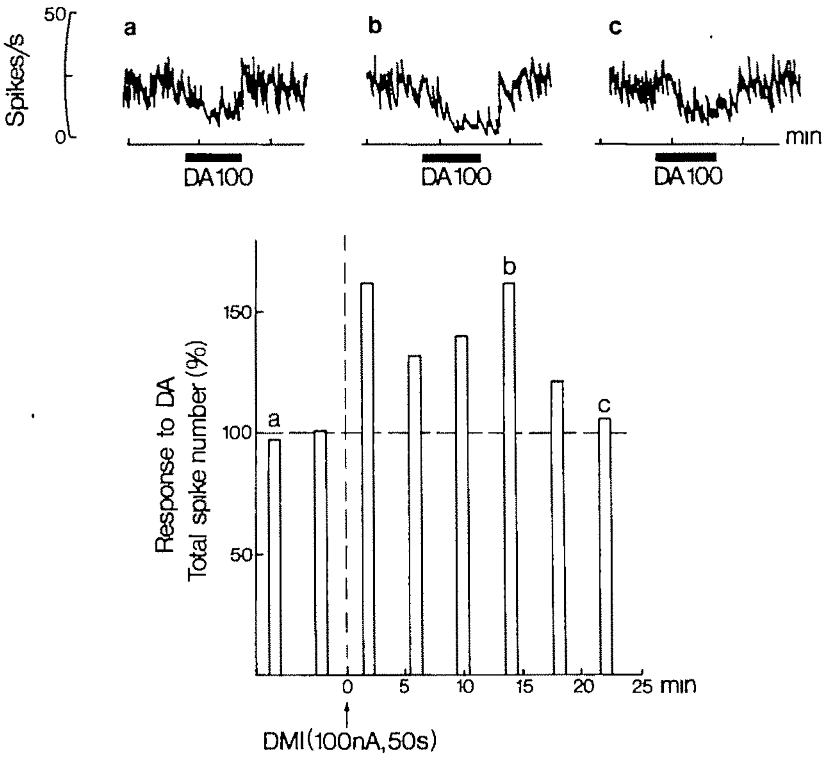

Figure 2 Potentiation of depressant responses of a single caudate neurone to dopamine (DA) by desipramine (DMI). Top of the figure shows excerpts from the ratemeter recording of the firing rate of the neurone (as in Figure 1) (a) Control response to dopamine; (b) potentiated response to dopamine 13 min after a brief application of desipramine (100 nA; 50 s); (c) recovery of the control response 21 min after the application of desipramine. Graph at the bottom shows the time course of the entire study (as in Figure 1).

of the control responses. In three of the cells in each group, both potentiation and antagonism were observed. The response was first antagonized following the application of desipramine, this antagonism was followed later by potentiation, and finally by recovery of the control response. In two cells no significant change in the size of responses could be observed after the application of desipramine. The degrees of potentiation or antagonism seen in each cell are summarized in Figure 5.

In another three cells the excitatory response was reversed into depression following the application of desipramine.

Depressant responses Both potentiation and antagonism of depressant responses to dopamine could be observed after a brief application of desipramine. Potentiation was seen in six cells, antagonism was seen in three cells. In one cell included in each group both antagonism and potentiation were observed. An example of the potentiation of the depressant response to dopamine by desipramine is shown in Figure 2. In one cell no significant change in the size of responses could be observed after the application of desipramine. The degrees of potentiation or antagonism seen in each cell are summarized in Figure 5.

In six other cells, the depressant response to dopamine was reversed into excitation following the application of desipramine.

Effect of desipramine on responses to noradrenaline

Excitatory responses Both potentiation and antagonism of excitatory responses to NA could be observed after a brief application of desipramine. Potentiation was observed in four cells, antagonism was seen in six cells. In one cell included in each group both antagonism and

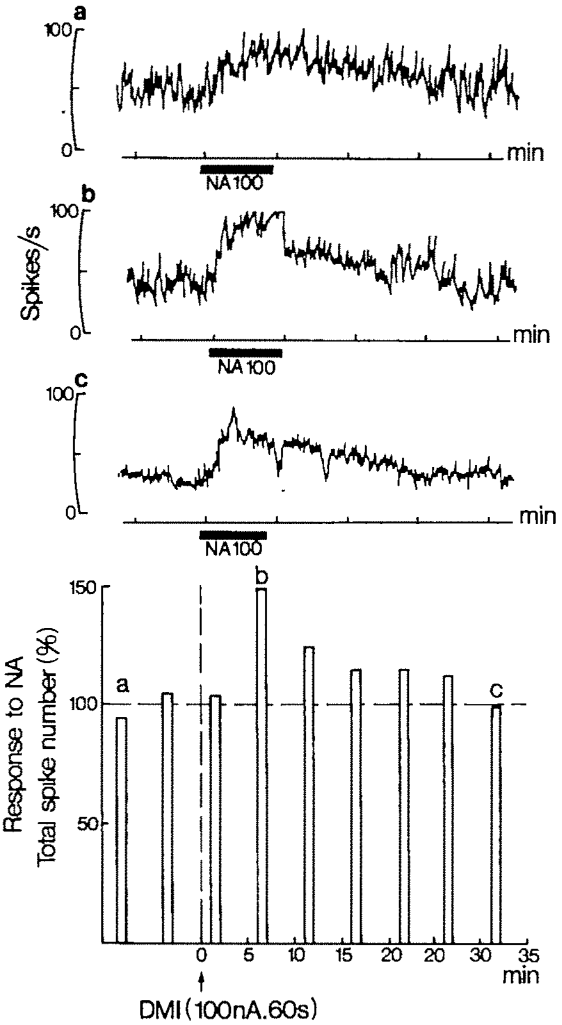

Figure 3 Potentiation of excitatory responses of a single caudate neurone to noradrenaline (NA) by desipramine (DMI). Top of the figure shows excerpts from the ratemeter recording of the firing rate of the neurone (as in Figure 1). (a) Control response to NA, (b) potentiated response to NA 6 min after a brief application of desipramine (100 nA, 60 s), (c) recovery of the control response 31 min after the application of desipramine. Graph at the bottom shows the time-course of the entire study (as in Figure 1).

potentiation were observed, the antagonism preceding the potentiation. An example of the potentiating effect of desipramine on responses to NA is shown in Figure 3. In one cell no significant change in the size of responses could be observed after the application of desipramine The degrees of potentiation or antagonism seen in each cell are summarized in Figure 5.

In one further cell the excitatory response to NA was reversed into a depression following the application of desipramine

Depressant responses Potentiation was seen in six cells depressed by NA, antagonism was observed in four cells. Both effects were seen in two cells included in both groups. In two cells no effect of desipramine could be observed. Figure 5 summarizes the degrees of potentiation or antagonism observed in each cell.

In another four cells the depressant response to noradrenaline was reversed into an excitatory one following the application of desipramine.

Effect of desipramine on responses to 5-hydroxytryptamine

Excitatory responses Potentiation was observed in four cells excited by 5-HT, antagonism was seen in three cells. In two cells in each group both antagonism and potentiation were seen, the antagonism preceded the potentiation. On two cells no effect of desipramine could be observed. The data are summarized in Figure 5.

Depressant responses In one cell, the depressant response to 5-HT was reversed into an excitation, following the application of desipramine.

Effect of desipramine on responses to acetylcholine

Both potentiation and antagonism of excitatory responses to ACh could be observed after a brief application of desipramine. Potentiation was observed in four cells, antagonism was seen in four cells. In one cell included in each group both antagonism and potentiation were seen, the antagonism preceded the potentiation An example of the potentiating effect of desipramine on responses to ACh is shown in Figure 4. In two cells desipramine had no significant effect. The data are summarized in Figure 5.

Effect of desipramine on responses to glutamate

The effect of desipramine on excitatory responses to glutamate was studied in 10 cells. The effects of glutamate on the firing rate were studied for 20-30 min following the application of desipramine. In none of the cells could any significant change be observed in the size of the responses to glutamate after the application of desipramine (See Figure 5).

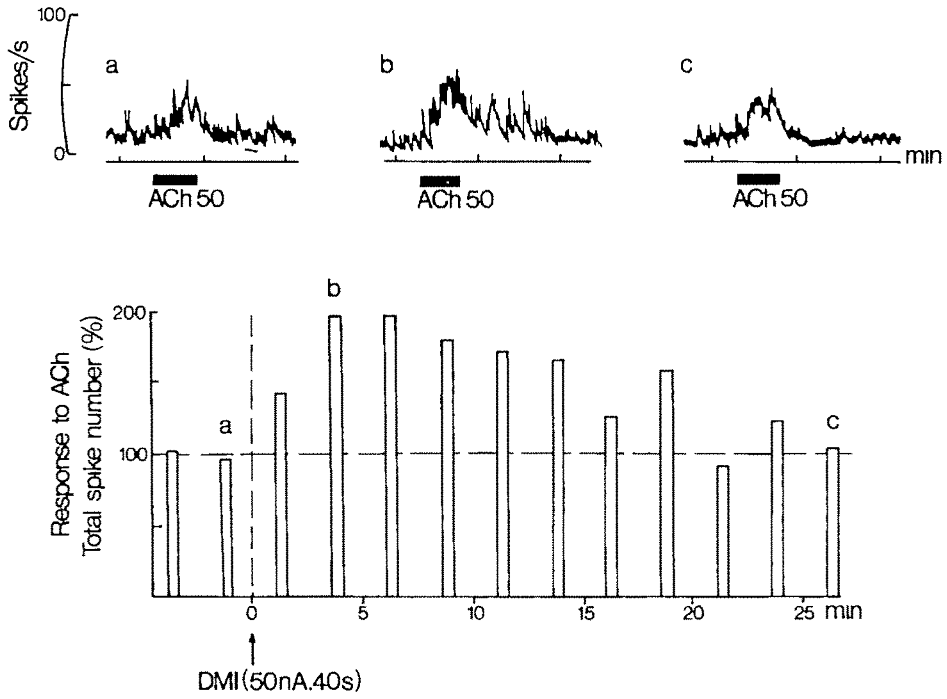

Figure 4 Potentiation of excitatory responses of a single caudate neurone to acetylcholine (ACh) by desipramine (DMI) Top of the figure shows excerpts from the ratemeter recording of the firing rate of the neurone (as in Figure 1) (a) Control response to ACh, (b) potentiated response to ACh 4 min after a brief application of desipramine (50 nA; 40 s); (c) recovery of the control response 26 min after the application of desipramine. Graph at the bottom shows the time course of the entire study (as in Figure 1).

Discussion

The results presented in this paper were obtained on spontaneously firing single neurones in the caudate nucleus of the rat. There have been some recent reports concerning the effects of dopamine (Gonzalez-Vegas, 1974, Woodruff, Elkhawad, Crossman & Walker, 1974; Siggins, Hoffer & Ungerstedt, 1974, Spencer & Havlicek, 1974) and of NA and ACh (Spencer & Havlicek, 1974) in this structure and in this species. Although Woodruff *et al* (1974) reported only depressant responses to dopamine in cells activated by homocysteic acid, other authors described the occurrence of both excitatory and depressant responses to DA (Gonzalez-Vegas, 1974; Siggins *et al.*, 1974, Spencer & Havlicek, 1974) and to NA (Spencer & Havlicek, 1974). We observed both excitatory and depressant responses to dopamine, NA, and 5-HT. Similarly, both excitatory and depressant responses to the monoamines have been described in the striate nucleus of the cat (Salmoiraghi & Stefanis, 1965, Bloom, Costa & Salmoiraghi, 1965; McLennan & York, 1967, York, 1970). A fair proportion of neurones in our material responded in a biphasic fashion to the monoamines (see Table 1). Biphasic responses to dopamine have been described in the striate nucleus of the cat (York, 1970), and of the monkey (York, 1972). The presence of biphasic responses and the observation of 'spontaneous response reversal' may suggest that both excitatory and inhibitory receptors to the same monoamine can co-exist on the same cell (Szabadi & Bradshaw, 1974). The high correlation between both the excitatory and depressant effects of dopamine and NA (compared to the lack of correlation between the effects of dopamine and 5-HT) (see Table 2) suggests that dopamine and NA may act at similar receptors on caudate neurones. Indeed, it is known that dopamine stimulates α-adrenoceptors in the periphery (Rossum, 1965).

ACh had a predominantly excitatory effect in our experiments. When responses to dopamine and ACh were compared on the same neurones, no significant correlation, either positive or negative,

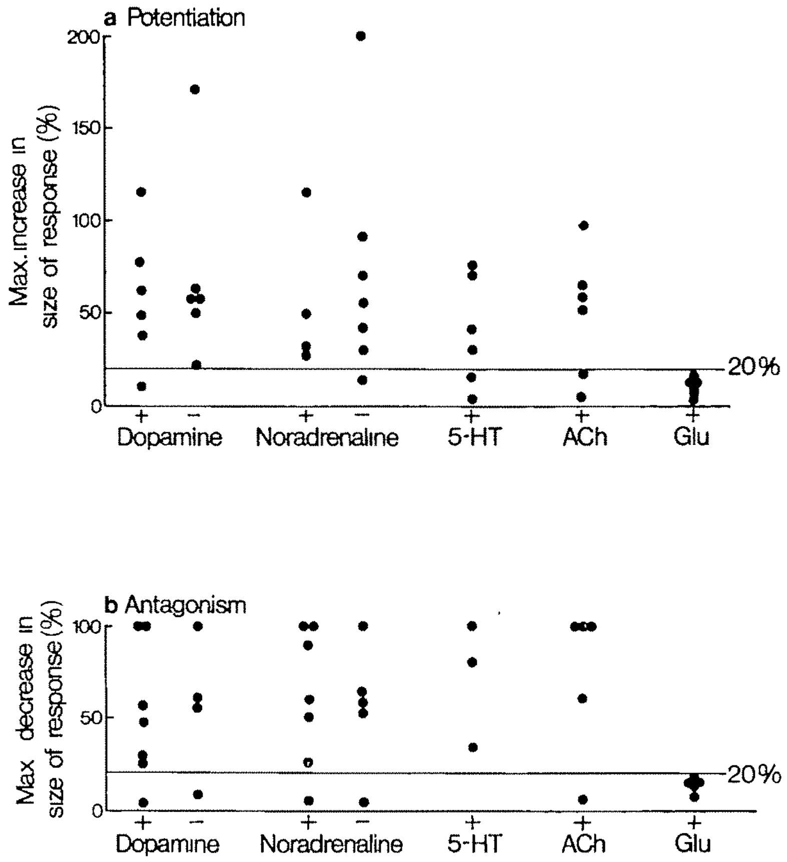

Figure 5 Summary of the effects of desipramine on neuronal responses to dopamine, noradrenaline, 5-hydroxytryptamine (5-HT), acetylcholine (ACh), and glutamate (Glu).

(a) Potentiation: each point shows the maximum potentiation observed on one individual cell (e.g. response b in Figure 1). +: excitatory responses, −: depressant responses. A response was regarded as potentiated if there was more than 20% increase over the size of the control response (see text).

(b) Antagonism: each point shows the maximum degree of antagonism observed on one individual cell. A response was regarded as antagonized if there was more than 20% decrease over the size of the control response (see text).

could be found (see Table 2). This would argue against the claim that dopamine and ACh have opposite effects in the caudate nucleus (Klawans, 1968, Yahr & Duvoisin, 1972).

Desipramine applied by microelectrophoresis had a dual effect on responses to monoamines and ACh: both antagonism and potentiation of the responses could be observed. Responses to glutamate were not affected. A dual effect of desipramine on responses to NA and 5-HT (Bradshaw *et al.*, 1974) and on responses to ACh (Bevan *et al.*, 1975a) has been described in the cerebral cortex of the cat.

The antagonism of responses to NA could be interpreted on the basis of the α-adrenoceptor blocking effect of desipramine (Türker & Khairallah, 1967). Since it is known that dopamine can stimulate peripheral α-adrenoceptors (Rossum, 1965), an α-receptor blocking action might explain the antagonism of responses to dopamine. The antagonism of responses to 5-HT may be explained on the basis of the anti-5-HT action of the antidepressants (Domenjoz & Theobald, 1959), whereas the antagonism of responses to ACh may reflect the antimuscarinic action of these drugs (Atkinson & Ladinsky, 1972).

It is more difficult to interpret the potentiating effects of desipramine. In the case of 5-HT, the most plausible explanation is uptake blockade.

There is indirect evidence that 5-HT terminals reach the caudate nucleus (Gumulka, Ramirez del Angel, Samanin & Valzelli, 1970), and that desipramine inhibits the uptake of 5-HT into brain tissue (Ross & Renyi, 1969). Uptake blockade, however, cannot explain the potentiation of responses to dopamine and NA by desipramine. It is known that both dopamine and NA are accumulated by an active uptake process in the striatum (Horn *et al.*, 1971); this uptake, however, is almost entirely unaffected by desipramine (Ross & Renyi, 1967; Horn *et al.*, 1971). An alternative explanation for potentiation could be that it reflects the blockade of masked, and functionally opposite, receptors on the post-synaptic neurone (Szabadi & Bradshaw, 1974; Bradshaw *et al.*, 1974). The blockade of masked receptors could also account for the prolongation of the recovery time of the potentiated responses often seen in our experiments (e.g. Figure 1), since the activation of the masked receptors may contribute to the termination of the response (Szabadi & Bradshaw, 1974). Our observation that the response to a monoamine was occasionally reversed after the application of desipramine can also be interpreted by this model. In this case, desipramine would block the dominant receptors selectively and thus reveal the effect of the masked receptors. However, since response reversal occurred spontaneously in a few cells, the causal relationship between the application of desipramine and the subsequent reversal of the response remains inconclusive.

It has been suggested that the potentiation of responses to ACh by desipramine could also be explained in terms of post-synaptic receptor blockade (Bevan *et al.*, 1975a). The occurrence of both excitatory and depressant responses to ACh in the caudate nucleus suggests the existence of two opposing receptor populations to ACh on caudate neurones, and thus the selective blockade of masked inhibitory receptors may account for the potentiation of excitatory responses. Moreover, there is evidence that the inhibition of cholinesterase cannot explain the potentiation of neuronal responses to ACh by desipramine (Bevan *et al.*, 1975a).

The results presented here show that desipramine can potentiate neuronal responses to dopamine in the caudate nucleus. It is possible that this action is responsible for the therapeutic efficacy of desipramine in Parkinson's disease. However, responses to ACh can also be potentiated by desipramine, and this might counteract the anti-Parkinsonian effect of desipramine. This may explain why a small dose of a potent cholinolytic drug has to be administered concurrently with desipramine in order to obtain the full therapeutic benefit from the anti-depressant in Parkinsonism (Yahr & Duvoisin, 1972).

This work was supported by the Scottish Home and Health Department and the Mental Health Trust and Research Fund. P.B. gratefully acknowledges the receipt of a research studentship from the Medical Research Council. Desipramine was a gift from Geigy (UK) Ltd. We are grateful to Mr R. Lamb for his technical assistance.

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(Received February 25, 1975.
Revised April 9, 1975.)

Br. J. Pharmac. (1975), 54, 295-300

INDOMETHACIN-INDUCED INCREASE IN NORADRENALINE TURNOVER IN SOME RAT ORGANS

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1 The effect of a prostaglandin synthesis inhibitor, indomethacin, on noradrenaline turnover rate in various rat tissues was determined from the product of the endogenous noradrenaline concentration and of the rate constant of (—)-[^{3}H]-noradrenaline decline after injection of the labelled amine in tracer doses.

2 Treatment of the rats with indomethacin (5 mg/kg p.o. five times during 2.5 days) increased noradrenaline turnover rate 32-36% in submandibular gland, spleen and heart, and 4-17% in epididymal and subcutaneous adipose tissue.

3 The extrapolated [^{3}H]-noradrenaline content at time 0 was greater in heart and adipose tissue of indomethacin-treated animals than in controls, while it was not significantly changed in spleen and salivary gland.

4 There was no apparent relationship between tissue concentrations of intravenously injected [^{14}C]-indomethacin and the effect of indomethacin on noradrenaline uptake and turnover rate in the different tissues.

5 Indomethacin treatment did not affect monoamine oxidase and catechol *O*-methyltransferase activities in the different tissues.

6 The results are consistent with the hypothesis that indomethacin increases noradrenaline turnover in the rat by blockade of a locally operating feed back inhibition of transmitter release by prostaglandins. However, additional effects, such as an increased impulse traffic, cannot be ruled out.

Introduction

There is considerable *in vitro* evidence that prostaglandins modulate noradrenaline (NA) release from nerve endings in many tissues (Hedqvist, 1973). The possibility that this mechanism might be physiologically important has received circumstantial support by the finding, that prostaglandin synthesis inhibitors, including indomethacin, do cause an increased NA release from cat spleen (Hedqvist, Stjärne & Wennmalm, 1971), rabbit heart (Samuelsson & Wennmalm, 1971; Chanh, Junstad & Wennmalm, 1972), and guinea-pig vas deferens (Hedqvist, 1973, Fredholm & Hedqvist, 1973a)

The demonstration that indomethacin can increase urinary excretion of NA in rats (Stjärne, 1972, Junstad & Wennmalm, 1972) seems to provide the only indication that this mechanism might also operate *in vivo*.

We have examined this question more directly by determining the effect of indomethacin on NA turnover, i.e. the time dependent decrease in [^{3}H]-NA content in different tissues following intravenous (i.v.) injection of [^{3}H]-NA. The results indicate that indomethacin increases NA turnover in a variety of rat tissues. Furthermore our results suggest that increased NA release from the sympathetic neurones, rather than altered inactivation of NA released, is the main cause of this effect.

Methods

Materials

Male Sprague-Dawley rats (Anticimex strain) weighing 200-300 g were used throughout the study. (—)-[7-^{3}H]-Noradrenaline (9.2 Ci/mmol) was obtained from the Radiochemical Centre, Amersham. Both [2-^{14}C]-indomethacin and unlabelled indomethacin were kind gifts of MSD International. S-adenosyl-(—)-[methyl-^{14}C]-methionine (500 μCi/mmol and [ethylamine-^{14}C]-dopamine hydrochloride (16 μCi/μmol), used in the enzyme assays, were obtained from the Radiochemical Centre, Amersham Unlabelled dopamine was obtained from Sigma (St Louis, U.S.A.). Other reagents and solvents were reagent grade from ordinary commercial sources.

Distribution of [^{14}C]-indomethacin

Six rats (200-260 g) kept at room temperature were given indomethacin, 5 mg/kg i.v. (in 0.5-0.8 ml 10% ethanol) containing 0.5 μCi[^{14}C]-indomethacin After 2.5 h the animals were killed by decapitation and the organs specified below rapidly dissected out, blotted on filter paper and weighed. The organs were then homogenized in an Ultra-Turrax homogenizer in 7 ml ethanol. Following centrifugation a 6 ml aliquot of the ethanol was mixed with 12 ml toluene scintillator and the [^{14}C]-radioactivity counted. The results are presented as μg of indomethacin, based on the specific activity of indomethacin in the injected solution

Indomethacin treatment

Indomethacin (5 mg/kg) was administered by means of a gastric tube twice daily for 2.5 days. Control animals received an equivalent amount of vehicle (1 ml/kg 1% ethanol). During the indomethacin treatment up to the time of death the animals were kept at 4° C

Noradrenaline turnover

Two hours after the last dose of indomethacin or vehicle the animals were given (−)-[^{3}H]-NA (10 μCi) in the tail vein and were killed by a blow on the head after an additional time of 30 min to 24 hours. The heart, submandibular glands, spleen and subcutaneous and epididymal adipose tissue were then quickly dissected out, blotted dry and weighed. The organs were homogenized and centrifuged and the supernatant adsorbed on alumina Noradrenaline in the eluates was assayed fluorimetrically (Euler & Lishajko, 1961) and radioactively measured in a liquid scintillation spectrometer, as described below.

Monoamine oxidase (MAO)-activity

MAO activity was determined according to Wurtman & Axelrod (1963). Tissues from indomethacin-treated and control animals of cold were homogenized in 10 volumes of cold isotonic KCl. After centrifugation at 4000 *g* for 30 min, 25 μl of the supernatant was taken for assay. [7-^{14}C]-Dopamine was used as the substrate Results are expressed as nmol (of dopamine deaminated) (mg protein)$^{-1}$ 30 min^{-1}

Catechol O-methyl transferase (COMT)-activity

COMT activity was determined essentially according to Giles & Miller (1967), using dopamine and S-adenosyl-(−)-[methyl-^{14}C]-methionine as substrates. The source of enzyme was 25 μl of the same extract as used for MAO determination. Incubations were performed at 37°C for 15 min after which time the reaction was stopped by 0.45 M borate buffer, pH 10

Protein was determined according to Lowry, Rosenbrough, Farr & Randall (1951) in 1 N NaOH after precipitation with 0.1% deoxycholate and 10% TCA.

For the determination of radioactivity of non-aqueous samples 1-6 ml of sample was shaken with toluene containing 4 g/l of Omnifluor (New England Nuclear, Boston) whereas aqueous samples were shaken with Instagel (Packard Instr. Co) and counted in a Packard Tri-Carb liquid scintillation spectrometer. Corrections for counting efficiency were made by means of external standardization

Results

Indomethacin treatment caused a marked reduction of body weight and of submandibular gland weight in animals kept at 4° C (Table 1). The same was true for animals kept at room temperature (not shown). The weight of subcutaneous adipose tissue of indomethacin-treated animals kept at 4°C was also lower than that of controls, but the difference may not be real since it is quite difficult to remove this tissue reproducibly.

Table 2 summarizes the results on NA-turnover in animals kept at 4° C. The assumption that endogenous NA content reflects a 'steady state' level was borne out by the finding that the NA content as measured 0 5, 4 or 24 h after the i v. administration of labelled NA did not differ significantly in any of the rat tissues examined As shown in the last column in the table the calculated rate of NA turnover in the tissues examined was increased by 4-36% in the indomethacin-treated animals In heart and in the two types of adipose tissue this was due to a significantly increased fractional turnover rate constant In the submandibular gland, on the other hand, this constant was significantly lowered coincident with a significant elevation of the 'steady state' NA content.

It is of interest to note that the extrapolated [^{3}H]-NA content at time 0 was higher in heart and adipose tissue from indomethacin-treated animals than in the same tissues from control animals (Table 2), while it was not significantly changed in spleen and salivary gland In the salivary gland we found no significant differences between control and indomethacin-treated rats in

Table 1 The effect of indomethacin treatment (5 mg/kg p.o. five times during 2.5 days) on the weight of whole rats and selected organs

	No of animals	*Body weight*	*Heart*	*Spleen*	*Submandibular gland*	*Epididymal adipose tissue*	*Subcutaneous adipose tissue*
Control	24	183 ± 5	0.86 ± 0.18	0.63 ± 0.12	0.48 ± 0.13	1.23 ± 0.29	1.22 ± 0.30
Indomethacin	21	160 ± 1	0.77 ± 0.13	0.66 ± 0.14	0.32 ± 0.12	1.21 ± 0.45	0.77 ± 0.26
		$P < 0.005$	NS	NS	$P < 0.005$	NS	$P < 0.001$

The initial weight of the animals was 200 g, and both control and indomethacin-treated animals were kept at +4° C during the period of treatment. Results are given in g ± s.e. mean. Statistical analysis according to Student's *t*-test for unpaired data.

Table 2 The effect of indomethacin treatment on tissue noradrenaline (NA) content (mean ±s.e. mean) and NA turnover in rats kept at +4° C

Tissue	*Indomethacin*	*Number*	*Uptake** $(10^3\ dmin^{-1}\ g^{-1})$	*Endogenous NA†* $(\mu g/g)$	*Rate‡* $k(h^{-1})$	*Turnover§* $(\mu g\ g^{-1}\ h^{-1})$	*Change in turnover* *(%)*
Heart	–	20	459 ± 55	0.52 ± 0.06	0.084 ± 0.003	0.044	+36
	+	20	621 ± 42	0.54 ± 0.06	0.110 ± 0.036	0.060	
			$P < 0.01$		$P < 0.05$		
Spleen	–	20	100 ± 42	0.60 ± 0.04	0.047 ± 0.018	0.028	+35
	+	20	120 ± 36	0.56 ± 0.06	0.068 ± 0.018	0.038	
Submandibular gland	–	20	201 ± 52	1.40 ± 0.07	0.089 ± 0.017	0.124	+32
	+	20	244 ± 49	2.32 ± 0.32	0.071 ± 0.003	0.164	
				$P < 0.01$	$P < 0.05$		
Subcutaneous adipose tissue	–	18	19 ± 4	0.039 ± 0.0006	0.070 ± 0.016	0.0027	+17
	+	20	33 ± 5	0.034 ± 0.0034	0.094 ± 0.015	0.0032	
			$P < 0.01$		$P < 0.01$		
Epididymal adipose tissue	–	18	12 ± 2	0.025 ± 0.0022	0.061 ± 0.013	0.0015	+4
	+	20	22 ± 3	0.020 ± 0.0033	0.079 ± 0.013	0.0016	
			$P < 0.01$		$P < 0.01$		

* 'Uptake' is presented as the intercept of the decline in [^{3}H]-radioactivity with the ordinate. Significance values based on Student's *t*-test.

† A correction (1/0.72) was made for losses on the alumina column. Significance based on Student's *t*-test

‡ Slope of decline curve (fractional turnover rate constant) by the method of least squares. Significance values based on analysis of covariance

§ Calculated according to the formula Turnover rate = kC_0, where k is the fractional turnover rate constant and C_0 is the steady state level of NA (Costa *et al.*, 1966).

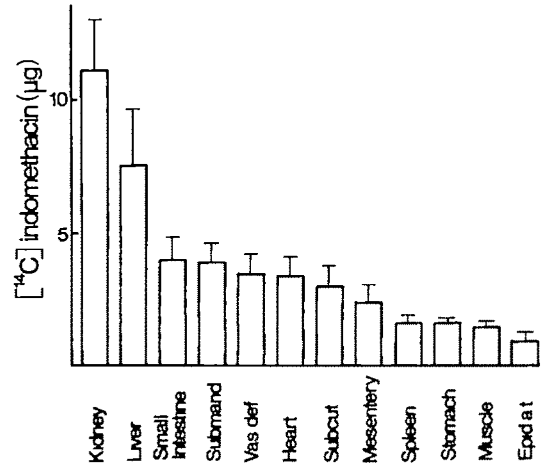

Figure 1 Distribution of indomethacin (μg) in rat tissues 2.5 h after i.v. administration of [^{14}C]-indomethacin (0.5 μCi/kg rat, specific activity 36 μCi/mmol). Mean values ± s.e. mean from six rats.

'uptake', whether this was expressed per unit weight of tissue or per μg of endogenous NA.

The data on [^{14}C]-indomethacin distribution between the tissues are presented in Figure 1. Our results are quite similar to those of Hucker, Zacchei, Cox, Brodie & Cantwell (1966) for those tissues that were examined in both studies. The plasma concentration was 13.2 μg/ml plasma 2.5 h after i.v. administration. There was no apparent relationship between the estimated tissue concentrations and the NA uptake level or turnover in the different tissues.

Indomethacin treatment lacked effect on MAO and COMT' activities of liver, heart, spleen submandibular gland and epididymal adipose tissue (Tables 3 & 4). When COMT activity in adipose tissue extracts was assayed under the same conditions as those used for the other tissues the amount of [^{3}H]-methoxy-tyramine was too low to permit determination, in agreement with the results of Stock & Westermann (1963).

Discussion

The present results demonstrate that indomethacin treatment increased the rate constant for efflux of [^{3}H]-NA in all tissues studied, except for submandibular glands. Since the concentration of endogenous NA did not vary during the period in which efflux of [^{3}H]-NA was followed (up to 24 h), measurement of the turnover rate of NA from the steady state relationship in which the rates of formation and efflux of NA are equal is justified (Costa, Boullin, Hammer, Voger & Brodie, 1966). From this it is evident that indomethacin treatment increased NA turnover in all studied tissues. The reason why NA turnover was increased in submandibular glands despite a decreased fractional release of [^{3}H]-NA is in all probability the loss of weight which occurred during the period of indomethacin pretreatment. This caused a relative increase of endogenous NA in this tissue while the content of NA per gland was unchanged.

The data demonstrate a 30-40% increase in turnover rate in heart, spleen and submandibular

Table 3 MAO activities in heart, liver, spleen, submandibular gland and epididymal adipose tissue from control rats and rats treated with indomethacin (5 mg/kg five times during 2.5 days).

	MAO activity (nmol(mg protein)$^{-1}$ 30 min^{-1})	
	Control	*Indomethacin*
Heart	38.0	38.1
Liver	16.1	14.6
Spleen	4.9	4.7
Salivary gland	6.6	7.4
Epid. adipose tissue	12.9	12.2

Means of duplicate determinations in two animals in each group.

Table 4 COMT activities in heart, liver, spleen and submandibular gland from control rats and rats treated with indomethacin (5 mg/kg five times during 2.5 days).

	COMT activity (nmol(mg protein)$^{-1}$ 15 min^{-1})	
	Control	*Indomethacin*
Exp. 1		
Heart	0.5	0.7
Liver	47.9	52.2
Spleen	8.0	8.3
Salivary gland	3.8	5.3
Exp. 2		
Heart	0.5	0.6
Liver	37.4	38.8
Spleen	5.4	3.6
Salivary gland	6.4	5.8

Means of duplicate determinations in two animals in each group.

gland on the third day of indomethacin treatment It is of interest that Stjärne (1972) found a similar increase in urinary NA excretion at that time. These results suggest that indomethacin causes a generalized increase in NA turnover.

The finding that the activities of the NA metabolizing enzymes were not affected by indomethacin tends to indicate that altered metabolic fate of the released noradrenaline is not a major cause of this increase in turnover. Clearly indomethacin might alter *in vivo* metabolism and the *in vitro* activity of enzymes differently. It is therefore interesting to note that Stjärne (1972) did not observe any change in the relative urinary excretion of (–)-[^{3}H]-NA as free intact amine by indomethacin treatment.

In the present experiments the extrapolated uptake of intravenously administered [^{3}H]-NA at time 0 was, if anything, increased by indomethacin. Volicer & Reid (1969) found that desipramine, an inhibitor of neuronal transmitter reuptake, actually decreased turnover. It is unlikely, therefore, that inhibition of the neuronal uptake mechanism could explain the observed effects with indomethacin. Since alterations in the disposition of NA is an unlikely explanation for the observed increase in turnover two main possibilities seem to remain: indomethacin causes either an increase in impulse flow or in the amount of NA released per impulse.

Considerable attention has been paid to the possibility that prostaglandins serve as physiological modulators of NA release (cf. Hedqvist, 1973). Aspirin-like drugs, such as indomethacin, which inhibit prostaglandin biosynthesis (Vane, 1971) might be used as tools to test this hypothesis. It should be pointed out in this context that homogenates of kidney from animals having received indomethacin according to the dosage schedule employed in this study converted only 3.2 ± 0.3% of 10 μg/ml [^{3}H]-arachidonic acid to prostaglandin E_2 compared to 7.0 ± 1.3% in controls (Fredholm, Hedqvist & Larsson, unpublished results). Thus there is evidence that indomethacin in this dose does inhibit prostaglandin biosynthesis. In a variety of tissues such as the cat spleen, rabbit heart and guinea-pig vas deferens, prostaglandins inhibit the release of overflow of NA per impulse and inhibitors of prostaglandin synthesis have the opposite effect (Hedqvist, 1973).

From these observations, it seems likely that indomethacin increases NA turnover in tissues and increases urinary excretion of NA by the removal of a prostaglandin mediated feed-back inhibition of NA-release per impulse.

Prostaglandin E_2 appears to have little effect on transmitter release (Fredholm & Hedqvist, 1973b) and indomethacin does not affect transmitter release during or after nerve stimulation in white adipose tissue (Fredholm & Hedqvist, 1975) Such an effect would require a prostaglandin concentration of the same order of magnitude as that required for inhibition of lipolysis.

Although controversial reports have appeared (Iliano & Cuatrecasas, 1971), the bulk of the recent evidence suggests that prostaglandins do not play a physiologically important role in the regulation of lipolysis in rat adipose tissue (Fain, Psychoyos, Czernik, Frost & Cash, 1973; Dalton & Hope, 1973; Fredholm & Hedqvist, 1974).

In the present study, NA turnover in adipose tissue was less affected by indomethacin than in the other tissues studied This finding, which cannot be explained in terms of uneven distribution of indomethacin between the different tissues, is compatible with the view that this tissue is devoid of a physiologically operative prostaglandin mechanism.

Even though NA turnover was affected least in adipose tissue, it was slightly elevated after indomethacin treatment, it was not possible to test the significance of this finding statistically. However, the possibility that indomethacin also increases impulse traffic in adrenergic neurones must be borne in mind. Animals treated with indomethacin are hypoglycaemic (Fredholm & Hedqvist, 1975) and hypoglycaemia stimulates sympatho-adrenal activity (cf. Himms-Hagen, 1967). It is not known, however, whether this will add to the stimulation induced by low environmental temperature.

In fat cells isolated from rats pretreated with indomethacin NA diminished the lipolytic response (Fredholm & Hedqvist, 1975). It is therefore conceivable, albeit remotely, that indomethacin treatment might cause a reduction in receptor sensitivity which could lead to reflex increase in sympathetic nerve activity and increased NA turnover (Dontas & Nickerson, 1957; Dairman, Gordon, Spector, Sjoerdsma & Udenfriend, 1968).

In conclusion, the present data have provided evidence that indomethacin treatment increases NA-turnover in the rat. It is unlikely that this effect is due to an alteration in NA disposition. Our results are in harmony with the hypothesis that the effect of indomethacin is due to blockade of a locally operating feed-back inhibition of transmitter release by prostaglandins, although additional explanations such as an increased impulse traffic, cannot be ruled out.

This study was supported by grants from Magnus Bergvalls Stiftelse and from the Swedish Medical Research Council, projects no 04X-4342 and 40X-2553.

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(Received January 27, 1975)

Br. J. Pharmac. (1975), **54**, 301-307

INHIBITION OF THE PULMONARY INACTIVATION OF PROSTAGLANDINS *in vivo* BY DI-4-PHLORETIN PHOSPHATE

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1 Inactivation of prostaglandin E_2 in the pulmonary circulation of rabbits *in vivo* was measured by comparing the hypotensive effects of doses given intravenously and intra-arterially.

2 Di-4-phloretin phosphate (DPP) 25-100 μg kg^{-1} min^{-1} inhibited the inactivation of prostaglandin E_2 in the pulmonary circulation.

3 These doses of DPP caused a marked shift to the left of the dose-response curve to prostaglandin E_2 given intravenously but did not affect the dose-response curve to prostaglandin E_2 given intra-arterially.

4 Inhibition of pulmonary inactivation of prostaglandins E_2 and $F_{2\alpha}$ caused marked potentiation of their gastrointestinal effects.

5 At these doses antagonism of the hypotensive action of prostaglandin E_2 by DPP was seldom seen but the gastrointestinal effect of prostaglandin $F_{2\alpha}$ was sometimes antagonized.

6 After treatment with DPP 100 μg kg^{-1} min^{-1}, high doses of prostaglandin were sometimes lethal.

Introduction

Prostaglandins of the E and F series are almost completely inactivated (90-98%) in one passage through the pulmonary circulation of cat, dog and rabbit *in vivo* (Ferreira & Vane, 1967a) and of guinea-pig isolated lungs *in vitro* (Piper, Vane & Wyllie, 1970). We have previously shown that inactivation of prostaglandins E_2 and $F_{2\alpha}$ *in vitro* can be inhibited by low concentrations of di-4-phloretin phosphate (DPP) and polyphloretin phosphate (Crutchley & Piper, 1974) At higher concentrations, polyphloretin phosphate selectively antagonizes certain prostaglandin actions *in vitro* and *in vivo* (Eakins, Karim & Miller, 1970, Villanueva, Hinds, Katz & Eakins, 1972).

The present study investigates whether DPP inhibits the pulmonary inactivation of prostaglandins *in vivo* Impairment of this inactivation would allow relatively more of an intravenous dose of prostaglandin to survive passage through the pulmonary circulation and so appear in increased amounts in the systemic circulation Thus, the systemic actions of prostaglandins would be expected to be potentiated. We have examined the effects of DPP on two actions of prostaglandins E_2 and $F_{2\alpha}$, these were changes in blood pressure and intestinal motility.

A preliminary report of this work was given to a meeting of the British Pharmacological Society (Crutchley & Piper, 1975).

Methods

Pulmonary inactivation of prostaglandin E_2

Male Dutch rabbits 1 8-2 5 kg were anaesthetized with pentobarbitone sodium 40 mg/kg intravenously A polyethylene cannula for intra-arterial infusions (after the lungs) was introduced retrogradely into the aortic arch via a common carotid artery. A similar cannula for intravenous infusions (before the lungs) was introduced into the superior vena cava via a jugular vein. Blood pressure was recorded from the remaining carotid artery or a femoral artery. Falls in blood pressure to prostaglandin E_2 were obtained by giving random 1 5 min infusions either intra-arterially or intravenously Since the resting blood pressures ranged between 80-110 mmHg, to allow for differences between individual animals, percentage falls in blood pressure were measured and dose-response curves were plotted Control measurements were made in the presence of a continuous infusion of 0.9% w/v NaCl solution (saline) (0.05 ml/min) into the remaining jugular vein. Test measurements were then made during an infusion of DPP in solution in saline (0.05 ml/minute).

Since bradykinin and noradrenaline are inactivated in a single passage through the pulmonary circulation (Ferreira & Vane, 1967b; Vane, 1969, Alabaster & Bakhle, 1972), we also investigated the action of DPP on the pulmonary inactivation of these compounds in rabbits *in vivo*. The method used estimated the inactivation by comparing the blood pressure responses to injections or infusions of bradykinin (depressor) or noradrenaline (pressor) by the intravenous and intra-aortic route.

Depressor and intestinal stimulant actions of prostaglandins E_2 and $F_{2\alpha}$

In these experiments the aortic cannula for intra-arterial infusion was not inserted. A midline incision was made in the abdomen, and the ileum exposed An enterotomy was performed and a small airfilled latex balloon was inserted. The balloon was inflated to a pressure of 30 mmHg, and ileal motility was measured by recording the intraluminal pressure via a S.E. Laboratory (SE4-82) pressure transducer. The pressure in the balloon always declined throughout the experiment irrespective of treatment. Blood pressure was recorded simultaneously from a carotid artery.

Prostaglandins were administered intravenously either by 1.5 min infusion or by injection. In the latter case, 0.1-0.25 ml prostaglandin solution was washed in with 0 3 ml saline. Dose-response curves to prostaglandins on intra-ileal and blood pressures were obtained during intravenous infusions of saline (control) and then DPP in saline (test).

Materials

All drugs were dissolved in saline before use. The following compounds were used: bradykinin (Sandoz), carbachol (Sigma), di-4-phloretin phosphate (Leo, A.B and Nelson Research & Development Co.), (−)-noradrenaline bitartrate (Sigma); prostaglandins E_2 and $F_{2\alpha}$ (Dr J.E. Pike, Upjohn Co., Kalamazoo, Mich.). In the cases where salts were used, doses of drugs are expressed in terms of free base.

Results

Effect of DPP on pulmonary inactivation of prostaglandin E_2

The depressor effects of prostaglandin E_2 were much less when it was given intravenously (before the lungs) compared with those when given intra-arterially (after the lungs). The difference gives a measure of the pulmonary inactivation of the prostaglandin (Figure 1). In the majority of experiments, the dose-response curve for intravenously administered prostaglandin was steeper than that obtained when it was given intra-arterially. This has been noticed by Bedwani & Marley (1975) and may be due to a variety of reasons. The most probable is that large intravenous doses of prostaglandin swamp the inactivating enzymes and so relatively more prostaglandin enters the systemic circulation as the dose is increased. Due to this non-parallelism at high doses, we calculated the inactivation of prostaglandins from falls in blood pressure not exceeding 15%. The mean control percentage removal was found to be 94.0 ± 0.6 ($n = 23$).

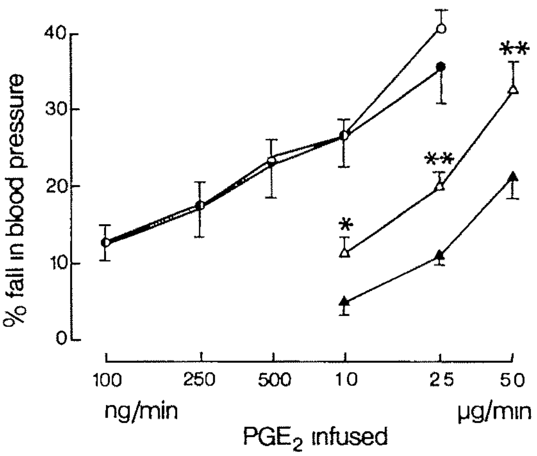

Figure 1 Inhibition of the pulmonary inactivation of prostaglandin E_2 (PGE_2) by di-4-phloretin phosphate (DPP). Random 1.5 min infusions of prostaglandin E_2 were given and the resulting falls in blood pressure noted. (●) Prostaglandin E_2 infused intra-arterially, during an intravenous infusion of saline (control)., (▲) prostaglandin E_2 infused intravenously (control), (o) prostaglandin E_2 infused intra-arterially, during an intravenous infusion of DPP 50 $\mu g\ kg^{-1}\ min^{-1}$ (test), (△) prostaglandin E_2 infused intravenously (test). Each point is the mean of up to eight experiments. Vertical bars show s.e. mean Test and control were done in the same animals. Significance levels were obtained by the paired t test. * $P < 0.01$, ** $P < 0.001$

DPP administered by intravenous injection (5-20 mg/kg) either had no observed effects, or produced a transient potentiation of the depressor effects of intravenously administered prosta-

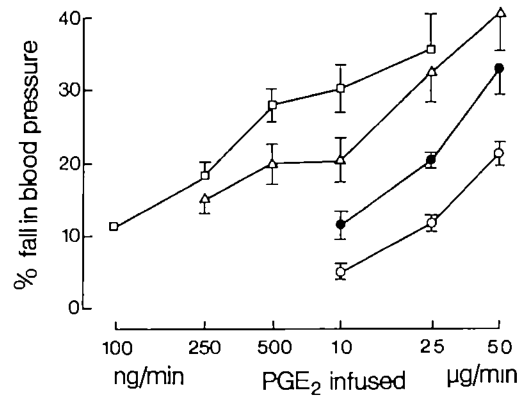

Figure 2 Dose-related effects of di-4-phloretin phosphate (DPP) on the pulmonary inactivation of prostaglandin E_2 (PGE_2) The percentage falls in blood pressure after random 1 5 min infusions of prostaglandin E_2 are shown. (●) Prostaglandin E_2 infused intravenously, during DPP 50 μg kg^{-1} min^{-1} intravenously (eight experiments), (△) prostaglandin E_2 infused intravenously during DPP 100 μg kg^{-1} min^{-1} (up to ten experiments); (○) prostaglandin E_2 infused intravenously during saline (control); (□) prostaglandin E_2 infused intra-arterially (control). These control measurements were obtained by combining the individual controls for DPP 50 or 100 μg kg^{-1} min^{-1} (up to 18 experiments). Vertical bars are s.e. mean.

glandin E_2. Continuous intravenous infusion of DPP 25 μg kg^{-1} min^{-1} had no effects on either blood pressure or the depressor response to prostaglandin given intra-arterially However, a small potentiation of the responses to intravenously administered prostaglandin occurred, as shown by a shift to the left of the dose-response curve (five experiments). However, only at a dose of 5 μg/min prostaglandin was the shift significant ($P < 0.02$). This shift in the absence of any effect on the responses to intra-arterially administered prostaglandin, suggests that DPP is acting to impair the pulmonary inactivation of the prostaglandin. At this concentration of DPP, the amount of prostaglandin surviving pasage through the pulmonary circulation increased 1.3 fold.

Continuous intravenous infusion of DPP 50 μg kg^{-1} min^{-1} again had no effect on either blood pressure or the responses to prostaglandin given intra-arterially but significantly potentiated the response to intravenous prostaglandin, as shown by a larger shift to the left of the dose-response curve (eight experiments) (Figure 1). The amount of prostaglandin surviving passage through the lungs increased 2.5 fold No increase of the effect of DPP with time was seen at these low doses (25 and 50 μg kg^{-1} min^{-1}) despite continuous infusion for up to 3 hours.

Continuous intravenous infusion of DPP 100 μg kg^{-1} min^{-1} had no effect on blood pressure before infusion of prostaglandin, but once this started, there was often a progressive fall in blood pressure as the experiment continued. This was particularly noticeable after high doses of prostaglandin, which may have been recirculating due to seriously impaired lung inactivation. Often this progressive decline in blood pressure after high doses of prostaglandin was fatal.

At this concentration DPP again had no effect on the blood pressure responses to prostaglandin given intra-arterially but caused a considerable potentiation of the responses to prostaglandin given intravenously (ten experiments). The amount of prostaglandin surviving passage through the lungs increased 16.4 fold (Figure 2). The animals often had diarrhoea after prostaglandin administration, presumably as a result of this increase. It was observed that the blood had an increased tendency to coagulate during intravenous infusion of DPP 100 μg kg^{-1} min^{-1} This occurred even after administration of heparin, 1000 iu/kg intravenously.

Thus, DPP itself had no effect on blood pressure, except at 100 μg kg^{-1} min^{-1} in the presence of prostaglandin. It had no significant effect on blood pressure responses to prostaglandin when given intra-arterially and hence had no noticeable prostaglandin inhibitory action at these doses It did cause a potentiation of the responses to prostaglandin administered intravenously, an effect which was dose-related. From this we conclude that DPP inhibits the pulmonary inactivation of prostaglandin E_2.

Reports from Leo AB indicate that DPP is 99.9% bound to plasma protein, so presumably the effective (unbound) concentration is a small fraction of the doses used.

In some experiments, the intravenous infusion of DPP was stopped and the time-course of recovery noted. This was quite rapid, pulmonary inactivation of prostaglandin often started to recover after 20-30 minutes.

Control percentage inactivations of bradykinin were 92.7 ± 1.5 and of noradrenaline 19 2 ± 4.6 (three experiments each). During a continuous infusion of DPP 100 μg kg^{-1} min^{-1} intravenously which would seriously impair the lung inactivation of prostaglandin E_2, the percentage inactivation of bradykinin was found to be 93.8 ± 2.3 and that of noradrenaline 15.8 ± 4 6 This shows that DPP has no significant effect on the pulmonary inactivation of bradykinin or noradrenaline.

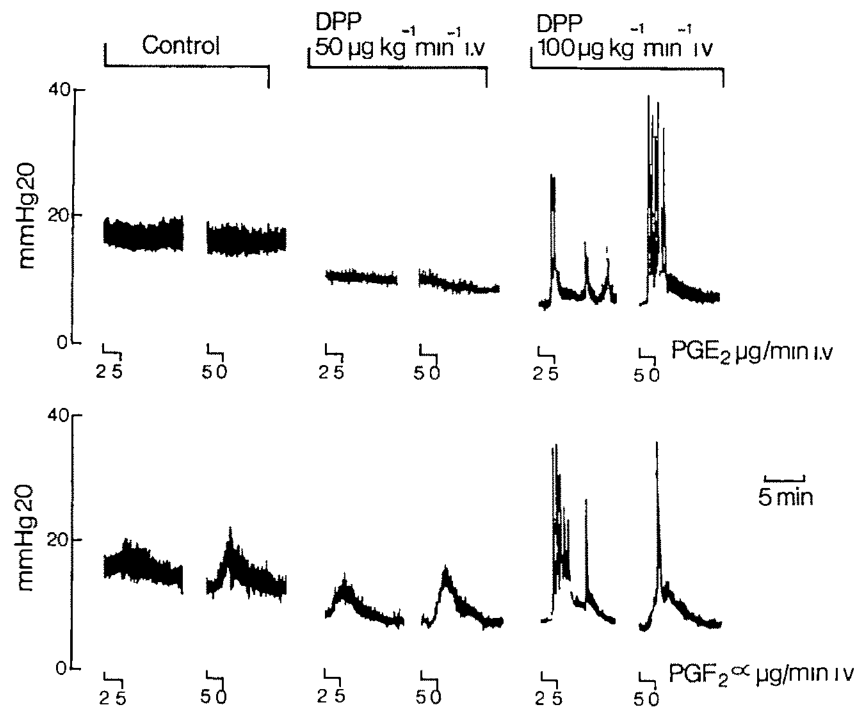

Figure 3 Potentiation by di-4-phloretin phosphate (DPP) of the effects of intravenous prostaglandins E_2 (PGE_2) and $F_{2\alpha}$ ($PGF_{2\alpha}$) on ileal motility. The effects of 1.5 min intravenous infusions of prostaglandin E_2 (upper trace) and $F_{2\alpha}$ (lower trace) on intra-ileal pressure are shown. Control measurements were made during a continuous intravenous infusion of saline. About 20 min after the start of a continuous intravenous infusion of DPP 50 µg kg^{-1} min^{-1}, slight potentiation of the responses to prostaglandin $F_{2\alpha}$ were seen During a continuous intravenous infusion of DPP 100 µg kg^{-1} min^{-1} potentiation of the effects of both prostaglandins occurred.

Effects of DPP on the depressor and intestinal stimulant actions of prostaglandins E_2 and $F_{2\alpha}$

Prostaglandin E_2 was administered by intravenous injection (1.0-10.0 µg, three experiments) or infusion (1.0-5.0 µg/min, one experiment). It caused dose-dependent falls in blood pressure and small increases in intra-ileal pressure. DPP 50 and 100 µg kg^{-1} min^{-1} had no effect on blood pressure or intra-ileal pressure but potentiated the falls in blood pressure and increased the rises in intra-ileal pressure caused by prostaglandin given intravenously (Figure 3). This effect of DPP was dose-related. DPP at these concentrations had no effect on the responses of the ileum to prostaglandin given intra-arterially (three experiments).

Prostaglandin $F_{2\alpha}$ was similarly administered by intravenous injection (three experiments) or infusion (four experiments). It was more potent than prostaglandin E_2 in increasing ileal motility. DPP 50 and 100 µg kg^{-1} min^{-1} potentiated the effects of prostaglandin $F_{2\alpha}$ given by infusion (one experiment) see Figure 3. In a further three experiments, DPP had no effect. DPP also potentiated the ileal response to prostaglandin $F_{2\alpha}$ administered by intravenous injection (one experiment). In a further two experiments, the responses to prostaglandin $F_{2\alpha}$ were depressed. This variable reaction of the ileum to prostaglandin $F_{2\alpha}$ in the presence of DPP may be due to direct antagonism by DPP of smooth muscle actions of the prostaglandin.

Prostaglandin $F_{2\alpha}$ had little or no effect on blood pressure except at high rates of infusion (25 0 µg/min) when it was depressor. During DPP 50 and 100 µg kg^{-1} min^{-1}, the highest doses of prostaglandin $F_{2\alpha}$ were strongly depressor. This may be due to impairment of the pulmonary inactivation of the prostaglandin allowing a higher effective concentration in the systemic circulation.

From these results we conclude that DPP potentiates the actions of intravenously administered prostaglandin E_2 on blood pressure and intra-ileal pressure It possibly potentiates the actions of prostaglandin $F_{2\alpha}$ on blood pressure and has a variable effect on the responses of the ileum to this prostaglandin.

The potentiation of responses to prostaglandin was probably not a non-specific cardiovascular change as DPP 100 μg kg^{-1} min^{-1} had no effect on either the depressor or intestinal stimulant actions of carbachol 1 0-3.0 μg intravenously (three experiments).

Discussion

The method used to determine the pulmonary inactivation of prostaglandin E_2 is a modification of that of Horton & Jones (1969) and has been used by other workers (Ueda, Hatanaka, Ito, Kokubu & Hamamura, 1973, Bedwani & Marley, 1974) It assumes that the depressor effects of prostaglandin E_2 are due solely to peripheral vasodilatation. Prostaglandins of the E series are potent vasodilators in a wide variety of vascular beds in a number of species, an effect which probably accounts for their systemic depressor actions (Horton, 1969).

The effects of the F-type prostaglandins on the circulation are complex. Prostaglandins $F_{1\alpha}$ and $F_{2\alpha}$ exert mild to moderate depressor actions in the cat and rabbit (Änggård & Bergström, 1963; Horton & Main, 1963) but are pressor in rat and dog, probably due to venoconstriction (Du Charme, Weeks & Montgomery, 1968). In cats, an increased pulmonary resistance, indicated by an increase in right ventricular pressure, may contribute to the depressor action (Anggård & Bergström, 1963). Hence, the pulmonary inactivation of prostaglandin $F_{2\alpha}$ was not determined by this method.

We have previously shown that DPP selectively inhibits the pulmonary inactivation of prostaglandins E_2 and $F_{2\alpha}$ by guinea-pig *in vitro* (Crutchley & Piper, 1974). The present studies show that DPP, when given by intravenous infusion, also inhibits the inactivation of prostaglandin E_2, and possibly $F_{2\alpha}$ by rabbit lungs *in vivo* The inactivation of bradykinin in the pulmonary circulation is enzymatic (Ryan, Roblero & Stewart, 1968) whereas that of noradrenaline is apparently due to an uptake mechanism (Vane, 1969). Since at the doses used DPP inhibits the inactivation of prostaglandins but not of bradykinin and noradrenaline it does not appear to be a general inhibitor of the inactivating functions of the lung.

Since the lungs are a major factor in the inactivation of circulating E and F prostaglandins, inhibition of this inactivation would be expected to potentiate the systemic effects of intravenously administered prostaglandin. We have shown that DPP does potentiate the depressor and intestinal stimulant actions of prostaglandins E_2 and $F_{2\alpha}$, although some direct antagonism of the actions of prostaglandin $F_{2\alpha}$ were seen. At 100 μg kg^{-1} min^{-1} DPP greatly inhibited the lung inactivation of prostaglandins, and the depressor effects of prostaglandin E_2 given intravenoulsy were potentiated to such an extent that they often proved fatal.

The increased coagulability of the blood observed during prostaglandin E_2 treatment in the presence of DPP may also have been due to recirculating prostaglandin. Prostaglandin E_2 may play a physiological role in haemostasis and blood clotting Although circulating blood normally contains little or no prostaglandin E_2 large amounts may be formed by platelets in response to thrombin generated by haemostasis or thrombosis (Silver, Smith, Ingerman & Kocsis, 1972). Prostaglandin E_2 also enhances the adenosine 5′-pyrophosphate-induced aggregation of platelets *in vitro* in a variety of species (Kloeze, 1967)

Both DPP and polyphloietin phosphate (PPP) are known to be specific antagonists of many of the smooth muscle actions of prostaglandins E_2 and $F_{2\alpha}$ *in vitro* (Eakins *et al*, 1970, Mathé, Strandberg & Åström, 1971, Eakins, Fex, Fredholm, Hogberg & Veige, 1973). PPP also antagonizes some of the smooth muscle actions of prostaglandins E_2 and $F_{2\alpha}$ *in vivo* (Villanueva *et al*, 1972). In these experiments, however, PPP was administered by injection In our experiments, injected DPP (5-20 mg/kg i.v) had little effect on the lung inactivation of prostaglandin E_2. It appears that DPP exhibits marked prostaglandin-inactivation inhibitory properties only if infused continuously We have observed that prostaglandin inactivation recovers fairly rapidly after cessation of the DPP infusion, recovery starts after about 20 minutes. Similarly, DPP washes out quickly from guinea-pig isolated perfused lungs (Crutchley & Piper, 1974). Villanueva *et al* (1972) observed that certain of the actions of prostaglandins were potentiated shortly after the injection of PPP. This may have been due to transient inhibition of the inactivating enzymes of the lungs.

Since the effects of low doses of DPP do not increase despite continuous infusion for up to 3 h, it would appear that the effective concentration of DPP in the blood remains essentially constant. This may be due to binding to plasma protein, or metabolism in the liver However, high doses (100 μg kg^{-1} min^{-1}) do appear to recirculate and cumulative effects were observed as the infusion of DPP continued

Villanueva *et al.* (1972) reported that PPP appears to be more potent as an antagonist of prostaglandin $F_{2\alpha}$ than of prostaglandin E_2 *in vivo.* Only certain of the effects of prostaglandin E_2 were antagonized by PPP. Our studies confirm this as at the doses used, direct antagonism by DPP occurred only to prostaglandin $F_{2\alpha}$.

The dual properties of DPP are interesting. On the one hand, inhibition of pulmonary prostaglandin inactivation would tend to potentiate the systemic effects of intravenously administered prostaglandins. On the other hand, antagonism of prostaglandin action would tend to reduce their effects. It appears that PPP inhibits some of the *in vivo* effects of prostaglandin E_2 more readily than others (Villanueva *et al.*, 1972). This raises the possibility of the development of an analogue, which would both increase prostaglandin survival on passage through the lung, and selectively antagonize unwanted prostaglandin actions. If this were possible, not only would the effective dose of intravenous prostaglandin be reduced, but the desirable effect would be preferentially potentiated, and thus the therapeutic ratio increased.

Our results emphasize the importance of the lungs in removing prostaglandins from the circulation. However, other sites especially the liver are capable of inactivating circulating prostaglandins (Vane, 1969). Prostaglandin dehydrogenase, the enzyme playing a key role in the biological inactivation of prostaglandins, has been found in lung, spleen, kidney, stomach, testicle and small intestine of the swine (Samuelsson, Granström, Gréen & Hamberg, 1971). As yet we have no information on the ability of DPP to inhibit the dehydrogenase in these tissues. However, since DPP did not potentiate the responses of the ileum to prostaglandins given intra-arterially either the dehydrogenase present in the ileum is relatively unimportant in the inactivation of circulating prostaglandins, or it is not inhibited by DPP, possibly due to the enzyme's location. Although the lungs are probably the most important site of prostaglandin inactivation, once this is impaired, other sites of inactivation may become relatively more important.

We wish to thank Dr J.E Pike of the Upjohn Company, Kalamazoo, Mich., U.S A for the gift of prostaglandins E_2 and $F_{2\alpha}$ and both Leo AB, Helsingborg, Sweden and Nelson Research and Development Company, Irvine, U.S.A. for DPP. We also thank the Medical Research Council and the Wellcome Trust for grants.

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(Received February 21, 1975.
Revised April 17, 1975)

Br. J. Pharmac. (1975), 54, 309-317

POTENCY AND SELECTIVITY OF METHYL ANALOGUES OF PROSTAGLANDIN E_2 ON RAT GASTROINTESTINAL FUNCTION

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1 The potency and selectivity of action of prostaglandin E_2 and its (15S)- or (15R)-15 methyl and 16,16 dimethyl analogues on gastrointestinal function have been studied in the rat.

2 The (15S)-15 methyl and 16,16 dimethyl analogues were 40 times as active as prostaglandin E_2 in inhibiting pentagastrin-stimulated acid secretion on intravenous administration to the anaesthetized rat, and 100 times as active on subcutaneous injection to the chronic fistula rat.

3 In antisecretory doses, the analogues, like prostaglandin E_2, caused bile reflux and, in higher doses, profuse diarrhoea.

4 The (15S)-15 methyl and 16,16 dimethyl analogues were at least 30 times as active as prostaglandin E_2 in causing changes in intestinal intraluminal pressure *in vivo*, but were equipotent on isolated smooth muscle.

5 In equivalent antisecretory doses, the methyl analogues had little effect on systemic arterial blood pressure and resting mucosal blood flow compared with prostaglandin E_2.

6 The (15R) methyl epimer administered parenterally had little effect on gastrointestinal function but brief acid incubation greatly increased its activity.

Introduction

Naturally-occurring prostaglandins of the E series inhibit gastric acid secretion in man (Classen, Koch, Bickhardt, Topf & Demling, 1971) and inhibit acid secretion and ulcer formation in animals (Robert, Nezamis & Phillips, 1968). However, their clinical usefulness is limited by side effects including cardiovascular effects and gastro-intestinal motility changes (Classen *et al.*, 1971) and by lack of antisecretory effects when administered orally (Horton, Main, Thompson & Wright, 1968). Recently, orally administered methyl analogues of prostaglandin E_2 (15-methyl and 16,16 dimethyl prostaglandin E_2) have been shown to inhibit gastric acid secretion in man (Karim, Carter, Bhana & Ganasan, 1973a, b; Robert, Nylander & Andersson, 1974). We have therefore compared the potency and selectivity of action of these analogues with the parent prostaglandin on several aspects of gastrointestinal function.

A preliminary report of this work has been presented to the British Pharmacological Society (Main & Whittle, 1974a).

Methods

Gastric acid secretion in the anaesthetized rat

The gastric lumen of the urethane-anaesthetized rat (200-250g body weight) was perfused with 0.9% w/v NaCl solution (saline, 0.2 ml/min) as previously described (Main & Whittle, 1973a) The acid output was determined in 0.5 ml aliquots of perfusate, collected at 20 min intervals, by titration to pH 7 with 0 01 M NaOH using an automatic titrator apparatus (Radiometer, Copenhagen), and expressed as output, μEquivalents/minute.

Gastric acid secretion in the unanaesthetized rat

The gastric lumen of the unanaesthetized rat was perfused with saline by a method similar to that described by Borella & Herr (1971). Following midline laparotomy in a pentobarbitone-anaesthetized (40 mg/kg s.c.) female rat, thin walled stainless-steel cannulae were implanted in the stomach, one in the pyloric antrum and one in the forestomach. The pyloric cannula was 15 mm long and 4.8 mm in diameter. The intra-abdominal portion had a 5 mm long polythene tip with a groove 1.5 mm from the end, into which the outer layer of gastric muscle was attached by a purse-string suture. Two close-fitting rings (15 mm diameter) of small-mesh nylon gauze were attached to the cannula, one at the gastric wall and the other to a groove 6 mm from the internal end. Connective tissue which formed on and between the gauze rings provided support and allowed only one third of the cannula to protrude externally. An external stainless-steel washer attached to a groove 4 mm from the outer end prevented the cannula from moving inwards. The cannula in the forestomach had a diameter of 2.5 mm but was similar to the pyloric cannula in all other respects.

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At least one month after surgery, a chronic fistula rat which had been starved for 18 h was placed in a Bollman-type restraining cage. After connecting the smaller cannula to a rubber catheter passed through adjustable slits in the cage floor and flushing the gastric lumen with warm saline to clear food particles, saline was perfused at 0.2 ml/min and the perfusate collected from the large cannula at 10 min intervals. Drugs were administered subcutaneously, or intravenously via a needle inserted into the tail vein.

Gastric mucosal blood flow

The [^{14}C]-aniline clearance technique was used to measure mucosal blood flow in the anaesthetized rat (Main & Whittle, 1973a). [^{14}C]-aniline was infused (0.033 μCi kg^{-1} min^{-1}, i.v.) and the gastric output and blood concentrations determined as previously described. The ratio of these values (clearance) gives an estimation of mucosal blood flow, which was expressed as % of basal values. Systemic arterial blood pressure was recorded and blood samples removed via a cannula inserted into a carotid artery. Drugs were administered through a cannula in a femoral vein.

Diarrhoea

Rats (190-230 g) were starved for 18 h in individual cages but allowed water. Following subcutaneous administration of the prostaglandin or saline, the number of rats in each group (each having at least 5 rats) exhibiting a mucoid diarrhoea within 1 h was noted. In control experiments, no wet faecal pellets or diarrhoea were observed.

Intestinal motility

Changes in intraluminal pressure *in vivo* were recorded from segments of the small intestine. Thin water-filled rubber balloons made from latex solution (Gerrard & Haig Ltd, Sussex), attached to polythene catheters, were introduced into the intestinal lumen via a small incision and tied in place avoiding large blood vessels Pressure changes were recorded on a Devices chart recorder (M2R) via a Statham pressure transducer (P23Db).

Isolated intestinal segments were suspended in oxygenated Tyrode solution at 37°C and contractions recorded isometrically via a Grass force displacement transducer (FTO3C).

Drugs

Prostaglandin E_2, (15S) or (15R)-15 methyl prostaglandin E_2 methyl ester and 16,16 dimethyl prostaglandin E_2 were dissolved in redistilled methanol and stored under nitrogen at −15°C. Aqueous solutions were prepared freshly when required after evaporation of the methanol, and adjusted to pH 6 with sodium bicarbonate before use.

Results

Inhibition of gastric acid secretion

Steady rates of submaximal gastric acid secretion were obtained in the urethane-anaesthetized rat after 60-80 min of an infusion of pentagastrin (0.3 μg kg^{-1} min^{-1} i v.). From preliminary experiments, the nature of the dose-response relationship of the prostaglandins in inhibiting acid output was determined. Near-maximal inhibition of acid secretion was obtained when prostaglandin E_2 (2 μg kg^{-1} min^{-1}) was infused intravenously for 40 min (Figure 1). A similar degree of inhibition, but of longer duration was obtained with a 40 min intravenous infusion of much lower doses of (15S)-15 methyl prostaglandin E_2 or 16,16 dimethyl prostaglandin E_2, 0.05 μg kg^{-1} min^{-1} (Figure 1). In further experiments, the same total dose of the analogues (2 μg/kg) gave a similar inhibition when infused over 20 min (Figure 2). From the results shown in Figures 1 and 2, it can be seen that the (15S) and 16,16 dimethyl analogues were at least 40 times more active than the parent prostaglandin as inhibitors of acid secretion. In contrast, the epimer (15R)-15 methyl prostaglandin E_2 caused only 60% inhibition of pentagastrin-stimulated acid secretion (from 1 29 ± 0.13 to 0.52 ± 0.1 μEquiv/min; $n = 3$) when infused for 40 min at a dose of 40 μg kg^{-1} min^{-1}. Thus it was at least 20 times less active than prostaglandin E_2 and 800 times less active than the (15S) analogue.

The effects of subcutaneous administration of the methyl analogues in the chronic fistula rat during resting acid secretion (2.8 ± 0.2 μEquiv/min, $n = 50$) are shown in Table 1. After allowing at least 90 min of perfusion for acid secretion to reach steady levels, three control collections were

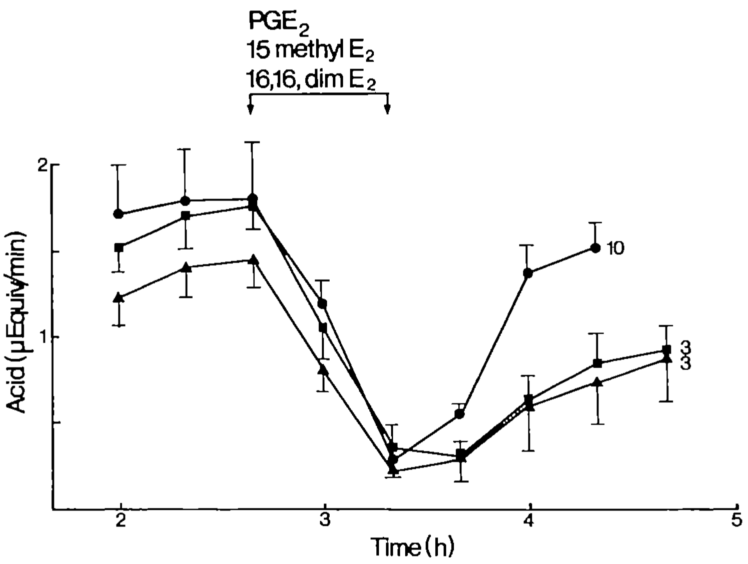

Figure 1 Effect of intravenously infused prostaglandin E$_2$ 2 μg kg^{-1} min^{-1} (PGE$_2$) (●) (15S)-15 methyl prostaglandin E$_2$ 0.05 μg kg^{-1} min^{-1} (■) and 16,16 dimethyl prostaglandin E$_2$ 0.05 μg kg^{-1} min^{-1} (▲) on pentagastrin-stimulated gastric acid secretion in the anaesthetized rat. Results are the mean of the number of experiments indicated on each curve. Vertical lines show s.e. mean.

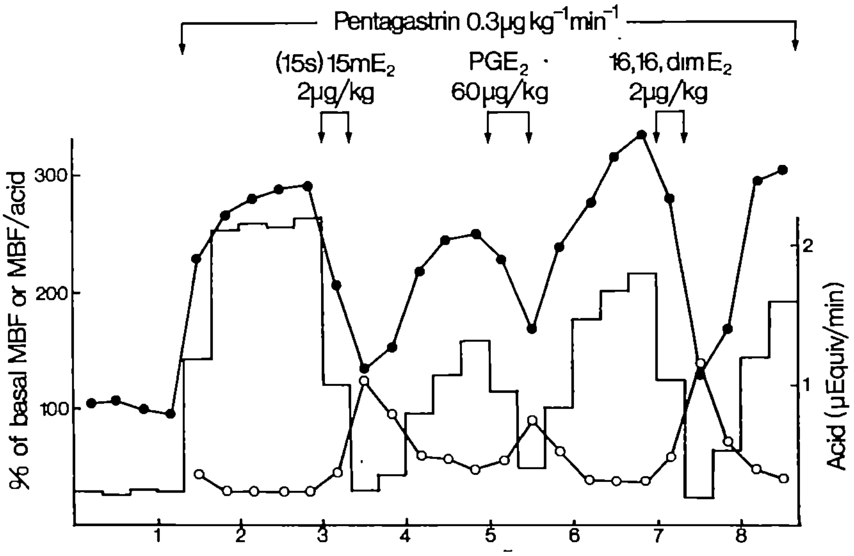

Figure 2 Effects of prostaglandin E$_2$ and methyl analogues on gastric acid secretion (⊓), mucosal blood flow (MBF ●) and the ratio of MBF to acid (○) during pentagastrin stimulation in the anaesthetized rat.

made prior to injection of saline or the prostaglandin (0.2-0.4 ml s.c.). The results in Table 1 were observed 20 min after injection and are expressed as % inhibition of resting acid output (the mean of the three control periods). The (15S) and 16,16 dimethyl analogues were approximately 100 times as active as prostaglandin E_2 when given subcutaneously. In two other experiments, the methyl analogues administered by intravenous injection had comparable effects.

In several experiments with high doses of prostaglandins, the gastric perfusate became alkaline ($pH > 7$). Furthermore, the clear perfusate became tinged yellow and the intensity and duration of this bile reflux was related to the dose of prostaglandin. The percentage of animals in each group in which bile reflux occurred is shown in Table 1.

The epimer, (15R)-15 methyl prostaglandin E_2 had only a slight inhibitory effect on acid output in subcutaneous doses of up to 4 mg/kg. However, after incubating this epimer at room temperature at pH 2 following the addition of 0.1 N HCl and readjusting to pH 6 with 0.1 N NaOH after 10 min, far smaller doses (10-25 μg/kg s.c.) caused a marked inhibition of acid secretion which was accompanied by bile reflux (Table 1) and diarrhoea.

Effects on gastric mucosal blood flow

Changes in mucosal blood flow ([^{14}C]-aniline clearance) during the stimulation and inhibition of gastric acid secretion are shown in Figure 2. Mucosal blood flow, which increased to a plateau during submaximal acid secretion stimulated by

Table 1 Effect of subcutaneously administered prostaglandin E_2 and its methyl analogues on gastric acid secretion and bile reflux in the conscious fistula rat

	Dose (μg/kg)	n	*Acid secretion (% inhibition)*	*Bile reflux (% incidence)*
Prostaglandin E_2	250	5	84 ± 7	100
	125	5	45 ± 13	80
(15S)-15 methyl E_2	10	3	82 ± 18	100
	2.5	5	80 ± 9	100
	1.25	5	46 ± 17	80
16,16 dimethyl E_2	2.5	5	84 ± 10	100
	1.25	6	35 ± 11	100
	0.625	4	19 ± 14	25
(15R)-15 methyl E_2	2500	4	11 ± 8	25
– after acid incubation	25	5	81 ± 19	100

Results, obtained from a colony of 12 rats used at intervals of at least one week, are expressed as mean with s.e., where *n* is the number of experiments.

Table 2 Ratio of mucosal blood flow (MBF) to acid secretion during pentagastrin-stimulated acid secretion, before and during administration of prostaglandin E_2 and its methyl analogues

	Dose ($\mu g\ kg^{-1}\ min^{-1}$)	*MBF/acid (% of basal)* *Pentagastrin*	*Pentagastrin + prostaglandin*	n
Prostaglandin E_2	2.0	40 ± 6	140 ± 21**	4
(15S)-15 methyl E_2	0.05	33 ± 3	106 ± 24**	3
16,16 dimethyl E_2	0.05	39 ± 4	95 ± 26*	3
(15R)-15 methyl E_2	40	39 ± 8	68 ± 14*	3

Results are expressed as mean with s.e. where *n* is number of experiments.
* $P < 0.05$, ** $P < 0.01$ (paired data, Student's *t* test)

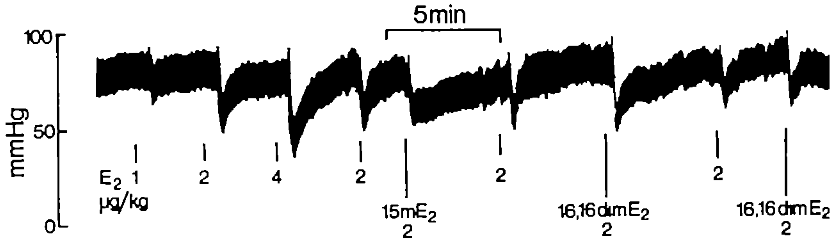

Figure 3 Effect of intravenous injection of prostaglandin E$_2$ (PGE$_2$), (15S)-15 methyl and 16,16 dimethyl analogues on systemic arterial blood pressure in the rat.

pentagastrin (0.33 µg kg^{-1} min^{-1}), fell during the inhibition of secretion by the prostaglandins. However, the ratio of mucosal blood flow to acid secretion, which reached steady values during pentagastrin stimulation (Figure 2), increased significantly during the administration of each prostaglandin (Table 2) indicating a primary inhibition of acid secretion.

During resting acid secretion and mucosal blood flow (acid saline, pH 2, being perfused to ensure adequate trapping of [^{14}C]-aniline) prostaglandin E$_2$ in a submaximal antisecretory dose (2 µg kg^{-1} min^{-1} i.v.) caused a rise in mucosal blood flow (Table 3). The (15S)-15 methyl and 16,16 dimethyl analogues in doses sufficient to cause complete inhibition of maximally-stimulated acid secretion (0.2 µg kg^{-1} min^{-1} i.v.) also caused an increase in resting mucosal blood flow, though of a lesser magnitude (Table 3).

Effects on systemic arterial blood pressure (BP)

Intravenous administration of an antisecretory dose of prostaglandin E$_2$ (2 µg kg^{-1} min^{-1}) caused a marked fall in BP which was maintained during the 40 min of infusion. In contrast, equivalent antisecretory doses of (15S)-15 methyl prostaglandin E$_2$ (0.05 µg kg^{-1} min^{-1}) caused only a small and transient fall in BP (Table 3). In four out of six experiments with 16,16 dimethyl prostaglandin E$_2$ there was little initial effect although BP subsequently rose gradually throughout the infusion. Intravenous infusion of the (15R) epimer (40 µg kg^{-1} min^{-1}) had no effect on BP (three observations).

The vasodepressor activities of the prostaglandins were further studied following single intravenous injections in the urethane-anaesthetized rat in six experiments. As is shown in Figure 3 the (15S) and 16,16 methyl analogues were slightly less active than prostaglandin E$_2$ in lowering BP although the effects were often more prolonged and sometimes showed tachyphylaxis.

Diarrhoea

The incidence of diarrhoea in groups of rats injected with the methyl prostaglandins is shown in Figure 4. The ED$_{50}$ (the dose producing diarrhoea within 1 h in 50% of the animals) was 7 µg/kg s.c., for (15S)-15 methyl prostaglandin E$_2$ and 17 µg/kg for 16,16 dimethyl prostaglandin E$_2$. Because of the large quantity of prostaglandin E$_2$ required for subcutaneous administration, only

Table 3 Effect of prostaglandin E$_2$ and its methyl analogues on resting mucosal blood flow (MBF) and systemic arterial blood pressure (BP)

	Dose (µg kg^{-1} min^{-1})	*Initial fall in BP (mmHg)*	*Resting MBF (% of basal)*	n
Prostaglandin E$_2$	2.0	34 ± 3	235 ± 41	4
(15S)-15 methyl E$_2$	0.05	8 ± 3	—	3
	0.20	16 ± 6	149 ± 18	6
16,16 dimethyl E$_2$	0.05	2 ± 6	—	3
	0.20	8 ± 4	136 ± 8	3

Results are the mean with s.e., where *n* is the number of experiments.

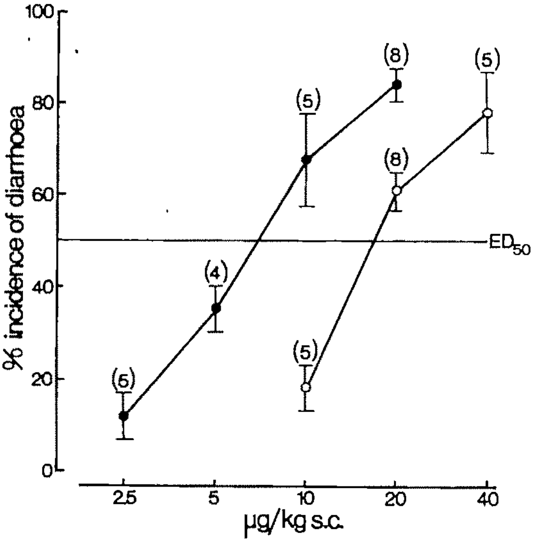

Figure 4 Incidence of diarrhoea in rats 1 h after subcutaneous injections of (15S)-15 methyl prostaglandin E_2 (●) and 16,16 dimethyl prostaglandin E_2 (○). Each point is the mean % incidence in several groups of rats when (*n*) is the number of groups. Vertical lines show s.e. mean.

three groups of rats at two dose levels (500 and 1000 μg/kg) were used; the ED_{50} was 820 μg/kg subcutaneously. The (15R)-15 methyl prostaglandin E_2 in subcutaneous doses of up to 5 mg/kg was inactive (five experiments) but after acid incubation as described above, doses of 10-20 μg/kg produced profuse mucoid diarrhoea (three observations).

Effects on intestinal motility

In a series of eight experiments *in vivo* intravenous administration of prostaglandin E_2 caused reproducible changes in intraluminal pressure of intestinal segments (upper and lower duodenum, jejunum and ileum) (Figure 5). The peak contraction occurred at a time when the fall in BP was returning towards resting levels. As shown in Figure 5a, the rapid rise in intraluminal pressure obtained with prostaglandin E_2 was also observed with the methyl analogues in comparable doses, but was followed by a second prolonged phase of intense motility. With lower doses, only the second phase of activity was observed (Figure 5b). Although comparison of potency with the parent prostaglandin is therefore difficult, these two methyl analogues were more than 30 times as active in inducing changes in intraluminal pressure.

The (15S) and 16,16 dimethyl analogues were approximately equipotent with prostaglandin E_2 on isolated gastrointestinal smooth muscle including rat forestomach strip, guinea-pig ileum and rabbit jejunum, as estimated by bracketing bioassay (two experiments on each tissue).

Discussion

Methylation at C-15 or C-16 protects prostaglandin E_2 from 15-hydroxy-prostaglandin

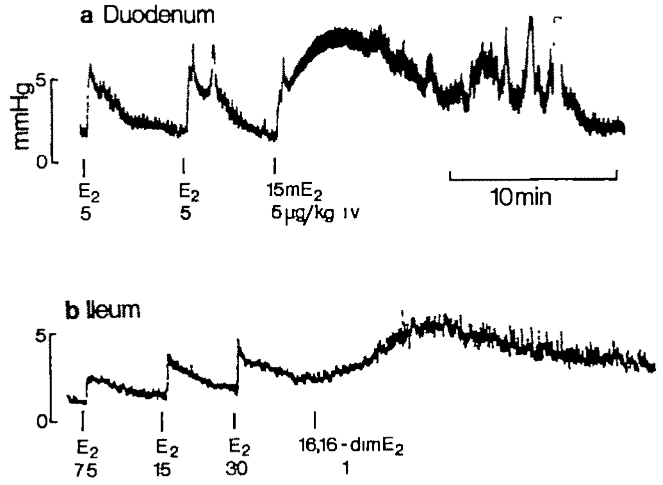

Figure 5 Effect of intravenous injections of prostaglandin E_2 and (a) its (15S)-15 methyl and (b) 16,16 dimethyl analogues on rat intestinal motility *in vivo*.

dehydrogenase (Weeks, Du Charme, Magee & Miller, 1973), an enzyme of prime importance in the metabolism and biological inactivation of prostaglandins (Samuelsson, Granström, Grēen & Hamberg, 1971). Thus the oral effectiveness and increased potency of the 15 methyl and 16,16 dimethyl analogues as inhibitors of gastric acid secretion in man (Karim *et al.*, 1973b; Robert *et al.*, 1974) may be due to decreased inactivation during and after absorption. Such an increase in potency does not, in itself, imply a change in the intrinsic activity of these analogues or in their spectrum of pharmacological actions. We therefore compared the potency and selectivity of these analogues with prostaglandin E_2 on several aspects of gastrointestinal function in the rat.

The (15S)-15 methyl and the 16,16 dimethyl analogues, administered intravenously, were approximately 40 times more active than prostaglandin E_2 in inhibiting pentagastrin-stimulated acid secretion in the anaesthetized rat, and their antisecretory effects were markedly prolonged. These results are comparable to those obtained in the Heidenhain-pouch dog after intravenous injection of these analogues (Robert & Magerlein, 1973). When administered subcutaneously to chronic fistulae rats, the methyl analogues were 100 times more potent than prostaglandin E_2 in inhibiting spontaneous secretion. This reduction in potency of prostaglandin E_2 on subcutaneous compared with intravenous injection may reflect metabolism during subcutaneous absorption.

In doses which inhibited gastric acid secretion by 50% or more, prostaglandin E_2 and the analogues caused a high incidence of duodenal reflux as indicated by the presence of bile in the gastric perfusate. Such reflux was unlikely to have contributed significantly to the reduction in acid output, which often returned towards control levels despite the continued presence of bile; moreover, inhibition was sometimes unaccompanied by bile reflux. The mechanism by which prostaglandins cause bile reflux has not yet been fully investigated but is likely to involve changes in duodenal motility and intraluminal pressure, as observed in the present investigation, coupled with changes in pyloric sphincter tone (Bertaccini, Impicciatore & De Caro, 1973). Evacuation of the gall-bladder cannot be a factor contributing to bile reflux in the rat, though it may be following prostaglandin administration in other species including man (Horton *et al.*, 1968).

The increased potency of the analogues in causing inhibition of acid secretion and bile reflux was also accompanied by increased potency on intestinal motility *in vivo* but not *in vitro*. It remains to be determined whether this increase in potency and the biphasic nature of the intestinal response to the methyl analogues can both be attributed solely to a decreased rate of inactivation. Our results on intestinal smooth muscle *in vitro*, which agree with those of other workers (Weeks *et al.*, 1973; Strand, Miller & McGiff, 1974) who compared the (15S)-15 methyl analogue to prostaglandin E_2, support the suggestion that the increased potency *in vivo* reflects decreased inactivation rather than a change in intrinsic activity.

In contrast, the methyl analogues were no more potent than prostaglandin E_2 as vasodepressors, as found by others (Weeks *et al.*, 1973; Strand *et al.*, 1974) using the (15S) analogue. In equivalent antisecretory doses, administered by intravenous infusion, the analogues had little detectable effect on the cardiovascular system compared with prostaglandin E_2, indicating a change in their selectivity of action. It has been suggested that the lack of increased vasodepressor potency of the methyl analogues may result from inactivation by enzymes other than 15-hydroxy-prostaglandin dehydrogenase (Weeks *et al.*, 1973). This would imply, however, that the resultant metabolites, while inactive on blood pressure, retain potent and long-lasting effects on gastro-intestinal function which we have observed in the present study.

The rise in mucosal blood flow per unit acid secretion during inhibition of secretion by the methyl analogues indicates that the changes in mucosal blood flow are secondary to changes in acid secretion. Furthermore, when acid secretion is completely inhibited by these methyl analogues, the vasodilator secretagogue, histamine, still increases mucosal blood flow (Main & Whittle, 1974b). During resting acid secretion the analogues caused a transient increase in mucosal blood flow indicating a direct vasodilator action on the mucosa, which is also seen with the parent prostaglandin. This agrees with our previous results using prostaglandins of E and A series (Main & Whittle, 1973b), though with the methyl analogues of prostaglandin E_2, there is no longer any parallelism between their effect on acid secretion and mucosal blood flow and their effects on blood pressure. It seems likely that the methyl analogues and the parent prostaglandins have the same mechanism of antisecretory action, possibly involving the adenyl cyclase-cyclic AMP system (Main & Whittle, 1974c).

The diarrhoea observed with low doses of the methyl analogues is likely to result from changes in intestinal motility; altered intestinal water and electrolyte secretion may also contribute to its fluid nature. Comparable effects were observed in other species including cat, guinea-pig and mouse (Main & Whittle, unpublished results). Although both analogues have greatly increased selectivity of action on gastric secretion compared with blood pressure, the results summarized in Table 4 show that in the rat, the increased potency of the (15S) analogue on acid secretion is associated with a comparable increase in potency on gastro-intestinal motility, as indicated by diarrhoea. The 16,16 dimethyl analogue appears to possess some selectivity of action, but its effects on motility are still very marked in doses which inhibit acid secretion. It is of interest that a high incidence of side effects, including diarrhoea, accompanied the termination of pregnancy by repeated administration of the (15S) and 16,16 dimethyl analogues (Ballard & Quilligan, 1974; Karim, Sivasamboo & Ratnam, 1974) in doses similar to those used to inhibit acid secretion in other studies (Karim *et al.*, 1973a b; 1974; Robert *et al.*, 1974).

The epimer (15R)-15 methyl prostaglandin E_2, is a potent and apparently selective inhibitor of gastric acid secretion in man when administered orally, but is inactive when injected intravenously (Karim *et al.*, 1973b). In the rat, the (15R) epimer is likewise a very weak inhibitor of acid secretion when administered intravenously or subcutaneously, being approximately 1000 times less active than the (15S) analogue. The observed activity, may, however, be accounted for by as little as 0.1% conversion to or contamination with the (15S) epimer. Brief incubation of the (15R) epimer in acid caused a very marked increase in potency on systemic administration, not only in inhibiting acid secretion, but in causing changes in gastrointestinal motility leading to bile reflux and diarrhoea. This result suggests that acid incubation converts the (R) to the (S) epimer and that this process, taking place in the acid environment of the stomach, can account for the oral effectiveness of the (R) epimer.

Our results indicate that, in the rat, the greatly increased potency of 16,16 dimethyl and (15S)-15 methyl prostaglandin E_2 in inhibiting acid secretion is accompanied by little or no increase in selectivity with respect to intestinal motility. If these analogues are to be of value in the treatment of peptic ulcer in man, very careful regulation of the dosage regimen will be required to avoid unacceptable gastro-intestinal effects on prolonged administration. The (15R) epimer, on activation by acid incubation, acquires the range of pharmacological actions characteristic of the (15S) analogue. Its therapeutic value compared with the other analogues will depend, therefore, on its site of action on the gastric mucosa and on the extent to which systemic absorption occurs or is an essential accompaniment to its antisecretory action.

We wish to thank Dr J.E Pike of the Upjohn Company, Kalamazoo, for supplying the prostaglandins, Miss Rosemary Hearn for expert technical assistance and the M.R C. for support.

Table 4 Comparison of the potency of prostaglandin E_2 and its methyl analogues in causing diarrhoea and inhibiting acid secretion

		ED_{50} (µg/kg, s.c.)	
	Diarrhoea	*Acid secretion*	*Selectivity ratio*
Prostaglandin E_2	820	138	5.9
(15S)-15 methyl E_2	6.8	1.4	4.8
16,16 dimethyl E_2	17.0	1.5	11.3

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(Received March 10, 1975)

Br. J. Pharmac. (1975), 54, 319-324

AN ANALYSIS OF THE DEPRESSOR RESPONSES TO HISTAMINE IN THE CAT AND DOG: INVOLVEMENT OF BOTH H_1- AND H_2-RECEPTORS

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1 The depressor responses to histamine, in anaesthetized cats and dogs, have been shown to involve both histamine H_1- and H_2- receptors.

2 In both species, histamine caused dose-dependent falls in blood pressure. The dose-response curve could be displaced to the right by administration of mepyramine 2.5×10^{-6} mol/kg i.v. The displacement was maximal with a dose-ratio of less than ten.

3 Metiamide alone, up to 2×10^{-6} mol kg^{-1} min^{-1}, had no significant effect on the histamine dose-response curve.

4 When administered in the presence of mepyramine, metiamide, 4×10^{-7} and 2×10^{-6} mol kg^{-1} min^{-1}, caused dose-dependent displacements to the right, of the histamine dose-response curve greater than could be achieved with mepyramine alone.

5 The results indicate the presence of both histamine H_1- and H_2-receptors in the cardiovascular system of the cat and dog. Both receptors produce a common response, i.e. a fall in blood pressure.

Introduction

In 1910, Dale & Laidlaw reported that histamine caused dose-dependent depressor responses in cats and dogs. The depressor responses to small doses of histamine can be reduced or abolished by the administration of H_1-receptor antagonists (Folkow, Haeger & Kahlson, 1948) although the responses to large doses of histamine are refractory to H_1-receptor antagonists (Staub, 1939; Folkow *et al.*, 1948).

Preliminary experiments with the histamine H_2-receptor antagonist, burimamide (Black, Duncan, Durant, Ganellin & Parsons, 1972) showed that the depressor responses to large doses of histamine, which are refractory to H_1-receptor antagonists, could be abolished by burimamide. These experiments are consistent with the involvement of both H_1- and H_2-receptors in the cardiovascular response to histamine in the cat and dog.

These observations have been extended using the selective H_1-receptor antagonist, mepyramine and the selective H_2-receptor antagonist, metiamide (Black, Duncan, Emmett, Ganellin, Hesselbo, Parsons & Wyllie, 1973) and the histamine depressor responses have been analyzed in terms of the receptors involved.

A brief account of some of these data has been presented to the British Pharmacological Society, March 1974 (Owen & Parsons, 1974).

Methods

Experiments were performed on cats of either sex, body weight 1.4-2.5 kg, anaesthetized by an intraperitoneal injection of sodium pentobarbitone, 60 mg/kg, and on beagle dogs of either sex, body weight 9.5-12 kg, anaesthetized by an intravenous injection of sodium pentobarbitone, 30 mg/kg. Supplementary doses of sodium pentobarbitone were given as necessary to maintain anaesthesia. In the cats the trachea was cannulated and the dogs were intubated with a cuffed endotracheal tube.

The right femoral artery was cannulated to measure systemic blood pressure with a Statham P23A blood pressure transducer. Blood pressure was monitored on a Devices M8 Electronic recorder. Drugs were administered via cannulae inserted into peripheral veins, usually the right femoral vein and right brachial vein.

Drugs

Histamine acid phosphate (B.D.H.), mepyramine maleate (May & Baker), phentolamine methane-

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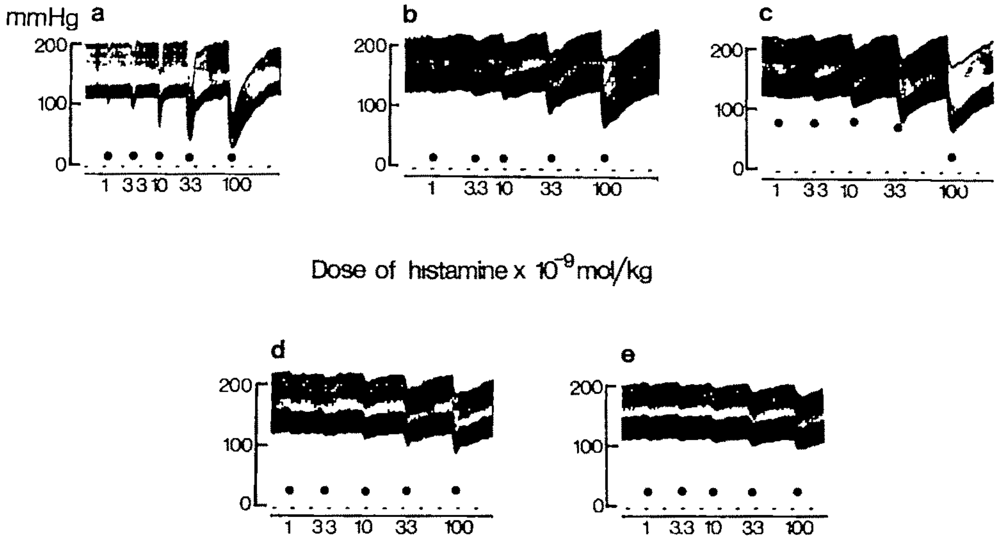

Figure 1 Anaesthetized dog blood pressure. (a) Histamine (●) caused dose-dependent falls in blood pressure. (b) The responses were reduced by mepyramine 2.5 x 10^{-5} mol/kg). (c) Increasing the dose of mepyramine to 5 x 10^{-5} mol/kg caused no further reduction in the histamine responses. (d and e) In the presence of mepyramine (5 x 10^{-5} mol/kg) metiamide (4 x 10^{-7} and 2 x 10^{-6} mol kg^{-1} min^{-1}) caused further dose-dependent reductions in the responses to histamine.

sulphonate (Ciba) and (±)-propranolol hydrochloride (I.C.I.) were prepared as solutions in 0.9% w/v NaCl solution (saline). Metiamide solutions were prepared by dissolving the base in a small quantity of 0.1 N HCl. The pH of the solution was then raised to 7 by the addition of 0.1 N NaOH and the solution finally made up to volume with saline.

Histamine was administered by injection, at intervals of 5 min, in a volume of 0.1 ml/kg and the venous cannula washed with 0.5 ml of saline. Dose-response curves were made by injection of increasing doses of histamine. Mepyramine, phentolamine and propranolol were administered as single, intravenous injections. Because of its shorter duration of action metiamide was administered by continuous intravenous infusion in volumes of 0.11-0.28 ml/minute. Infusions were made for 30 min prior to repeating the histamine doses and continued during dosing.

Results

Anaesthetized dogs

Histamine administered intravenously caused dose-dependent falls in systemic blood pressure (Figure 1), the threshold dose was about 1 x 10^{-10} mol/kg. No attempt was made to obtain maximal depressor responses as the blood pressure did not always return to the pre-injection level in control dogs after doses in excess of 1 x 10^{-7} mol/kg. Nevertheless a linear dose-response relationship could be obtained over the dose-range 1 x 10^{-9}-1 x 10^{-7} mol/kg (Figures 1 and 2).

Administration of mepyramine (2.5 x 10^{-6} mol/kg) caused a parallel displacement to the right of the histamine dose-response curve (Figure 2). Increasing the dose of mepyramine to 2.5 x 10^{-5} or 5 x 10^{-5} mol/kg did not significantly displace the dose-response curve further to the right. Two groups of experiments were made in which the maximum displacement of the dose-response curves occurred with a dose-ratio of 3.2 (3.1-3.5, 95% confidence limits), in one group and a dose-ratio of 4.2 (2.3-7.4) in the second group. When the maximum blocking effect of mepyramine had been achieved, infusions of metiamide (4 x 10^{-7} or 2 x 10^{-6} mol kg^{-1} min^{-1}) caused further dose-dependent displacement of the dose-response curve to the right (Figures 1 and 2). The displacements of the dose-response curves by metiamide in the experiments were not parallel, the slope of the curves being slightly less steep than the slope of the dose-response curve from untreated animals. The reason for this was that because of the long duration of the experiments, and because of the hypotensive effects of mepyramine (2.5 x 10^{-5} mol/kg), the resting blood

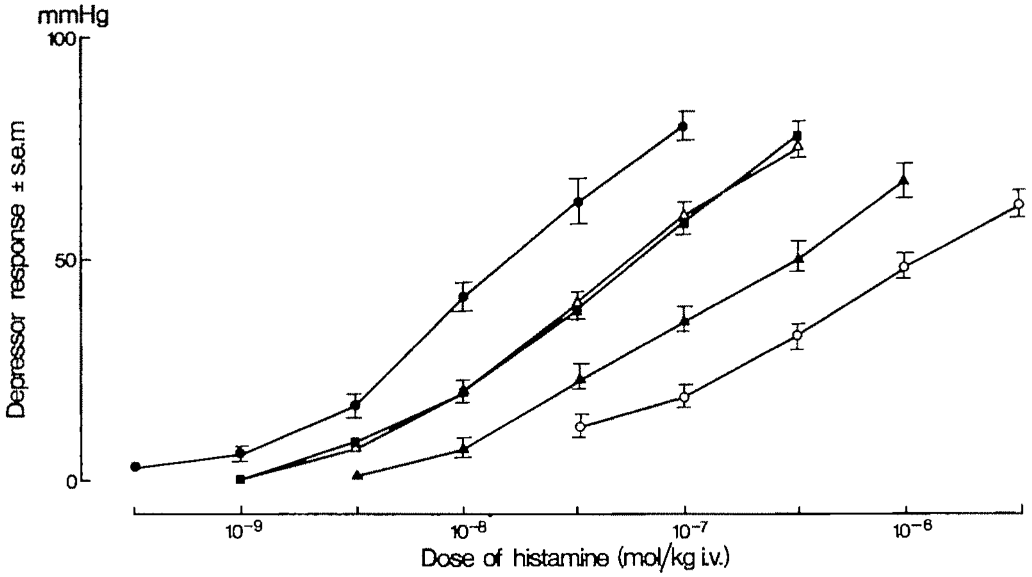

Figure 2 Anaesthetized dog blood pressure. Mean dose-response curves to histamine from five dogs. Responses to histamine prior to treatment with antagonists are indicated by (●). The dose-response curve was displaced to the right by mepyramine 2.5 x 10^{-4} mol/kg (■). Increasing the dose of mepyramine to 2.5 x 10^{-5} mol/kg caused no further displacement of the dose-response curve (△). Metiamide 4 x 10^{-7} mol kg^{-1} min^{-1} (▲), and 2 x 10^{-6} mol kg^{-1} min^{-1} (○), in the presence of mepyramine caused further dose-dependent displacements of the histamine dose-response curve.

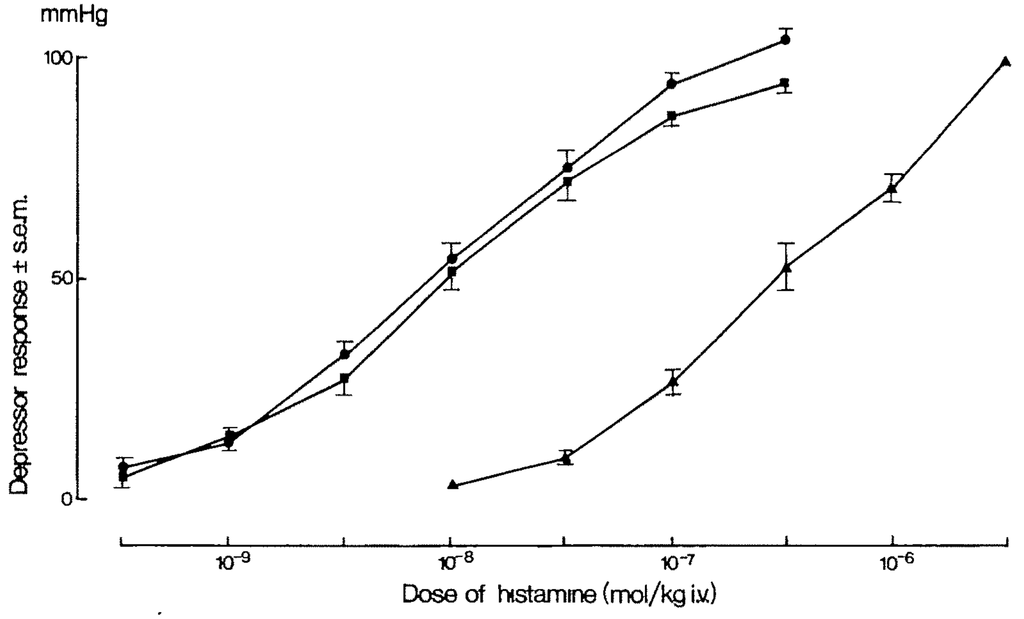

Figure 3 Anaesthetized dog blood pressure. Mean dose-response curves to histamine from five dogs. Responses to histamine prior to treatment with antagonists are indicated by (●). The dose-response curve was unaltered by metiamide 2 x 10^{-6} mol kg^{-1} min^{-1} (■). In the presence of metiamide, mepyramine 2.5 x 10^{-6} mol/kg (▲) caused displacement of the dose-response curve.

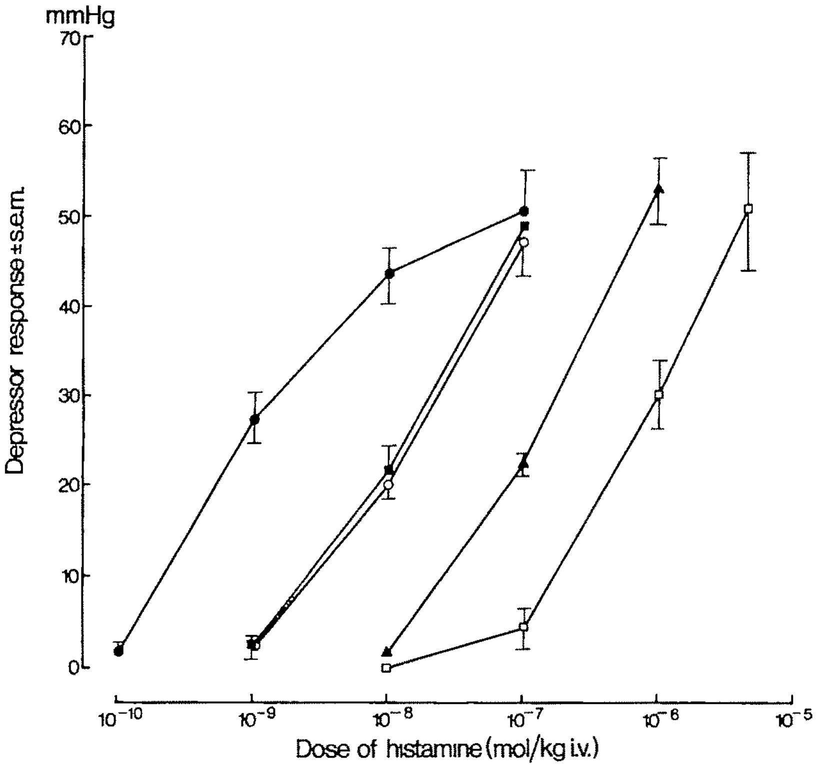

Figure 4 Anaesthetized cat blood pressure. Mean dose-response curves to histamine from five cats. Responses to histamine prior to antagonists are indicated by (●). The dose-response curve was displaced to the right by mepyramine 2.5 x 10^{-5} mol/kg (■). Mepyramine 5 x 10^{-5} mol/kg caused no further displacement of the dose-response curve (○). Metiamide 4 x 10^{-7} mol kg^{-1} min^{-1} (▲) and 2 x 10^{-6} mol kg^{-1} min^{-1} (□) caused further dose-dependent displacement, to the right, of the dose-response curve.

pressure at the end of the experiments was usually lower than at the start. Four additional short experiments were done to avoid this problem. Dose-response curves were obtained to three doses of histamine in untreated animals, and again after mepyramine (5 x 10^{-6} mol/kg), in order to achieve a maximum shift in the dose-response curve, and during the infusion of metiamide (4 x 10^{-7} mol kg^{-1} min^{-1}) plus mepyramine (5 x 10^{-6} mol/kg). The doses of histamine were chosen from the earlier experiments to lie on the linear part of the dose-response curve. In these shorter experiments there were parallel shifts in the dose-response curves after mepyramine, dose-ratio 7.1 (5.7-8.9, 95% confidence limits) and after mepyramine plus metiamide, dose-ratio 16.3 (13.3-20.1, 95% confidence limits).

Metiamide alone (2 x 10^{-6} mol kg^{-1} min^{-1}) had little or no effect on the histamine dose-response curve (see Figure 3). Administration of mepyramine, (2.5 x 10^{-6} mol/kg) during the continued infusion of metiamide, caused a parallel shift in the histamine dose-response curve with a dose-ratio of 42.0 (31.8-52.3, 95% confidence limits) i.e. the shift in the dose-response curve caused by mepyramine (2.5 x 10^{-6} mol/kg) was approximately 8-10 times greater during the infusion of metiamide than in its absence.

Anaesthetized cats

Histamine administered intravenously caused dose-dependent falls in blood pressure over the dose-range 1 x 10^{-10} to 1 x 10^{-7} mol/kg. In contrast to dogs, maximal depressor responses could usually be obtained after histamine (1 x 10^{-7} mol/kg) with recovery of the resting blood pressure to the pre-injection level. In most cats, the depressor responses to the larger doses of histamine were followed by secondary pressor responses which were due to release of catecholamines from the adrenal medulla and other chromaffin tissues (Burn & Dale, 1926). Unlike the depressor responses which were reproducible, the magnitude of the secondary pressor responses varied when the same dose of histamine was given repeatedly.

As in the dog, mepyramine (2.5×10^{-6} mol/kg) displaced the histamine depressor dose-response curve to the right and abolished the secondary pressor responses. However and in contrast to dogs, the displacement of the histamine dose-response curve in the cat was not parallel, the slope of the curve being steeper after mepyramine than in the untreated animals (Figure 4). The change in the slope of the dose-response curve appeared to be due to the abolition of histamine-induced catecholamine release by mepyramine (Emmelin & Muren, 1949). In four experiments in which the effects of the catecholamines were prevented by pretreatment with phentolamine, (1.6×10^{-5} mol/kg) and propranolol (8.4×10^{-6} mol/kg) the displacement of the histamine dose-response curve caused by mepyramine was parallel. A maximum shift could be achieved by mepyramine (2.5×10^{-6} mol/kg) with a dose-ratio of 8.6 (4.7-15.8, 95% confidence limits). When a maximum shift in the histamine dose-response curve caused by mepyramine had been achieved, metiamide (4×10^{-7} or 2×10^{-6} mol kg^{-1} min^{-1}) caused further dose-dependent displacements of the histamine dose-response curve (Figure 4). As in the dog metiamide (2×10^{-6} mol kg^{-1} min^{-1}) alone had no effect on the histamine dose-response curve although subsequent administration of mepyramine (2.5×10^{-6}, 8.3×10^{-6} and 2.5×10^{-5} mol/kg) produced dose-dependent displacements of the dose-response curve to the right

Discussion

Administration of histamine to cats and dogs results in a fall in systemic blood pressure The purpose of this study was to analyze the depressor response to histamine in terms of the receptors involved. Prior to our study Staub (1939) had shown that the depressor response to a large dose of histamine, 6.6×10^{-7} mol/kg, was refractory to histamine (H_1-receptor) antagonists of the Fourneau series. Subsequently Folkow *et al.* (1948) observed that the responses to small doses of histamine could be abolished by diphenhydramine and analogues of diphenhydramine but confirmed that the responses to larger doses of histamine were refractory to histamine (H_1-receptor) antagonists Folkow *et al.* (1948) suggested that 'there are two types of receptors sensitive to histamine, only one of which can be blocked by benadryl (diphenhydramine) and related compounds'. Our findings establish the involvement of the two types of histamine receptor in the depressor response to histamine in the dog and cat.

In 1972 Black *et al.*, showed that, in the cat, histamine responses that were refractory to H_1-receptor blockade could be blocked by H_2-receptor blockade, indicating the presence of cardiovascular H_2-receptors and their involvement in these depressor responses.

In both species, mepyramine caused displacement to the right of the histamine dose-response curve, whereas metiamide alone did not displace the dose-response curve. In the dog, the displacement of the dose-response curve was parallel but it was not in the cat, unless there had been prior blockade of the responses to catecholamines released by histamine (Burn & Dale, 1926). The release of catecholamines is reduced or abolished by mepyramine (Emmelin & Muren, 1949) In untreated animals the catecholamines opposed the depressor response to histamine and the elevated catecholamine levels often persisted to elicit a secondary pressor response. Mepyramine simultaneously abolished the catecholamine release and reduced the depressor response to histamine It is not, therefore, surprising that the displacement of the dose-response curve caused by mepyramine was not parallel unless there was prior adrenoceptor blockade. There are no quantitative data on catecholamine release in cats and dogs but it appears likely that greater release occurs in the cat than in the dog.

The displacement of the dose-response curves by mepyramine reached a maximum and this was achieved with similar dose-ratios in both dogs and cats. In both species once the maximum effect of mepyramine had been achieved further displacement of the histamine dose-response curve could be achieved by administration of metiamide. The displacement of the dose-response curve caused by metiamide was parallel indicating the competitive nature of histamine H_2-receptor blockade by metiamide.

From these experiments it is clear that large displacements of the histamine dose-response curves can only be achieved by simultaneous administration of both an H_1-receptor antagonist and an H_2-receptor antagonist. Ariëns, van Rossum & Simonis (1959) considered the theoretical dose-response curves likely to result from the interaction of one agonist with two independent receptor systems producing effects by means of a common effector system This theory should apply to the situation studied in our experiments. One agonist, histamine, interacts with two independent receptors, histamine H_1- and H_2-receptors, to produce a common effect, a fall in blood pressure. As far as could be measured, the maximum responses which followed interaction with either receptor were comparable. Under these circumstances Ariëns *et al.* (1959) found that the shape of the dose-response curves varied with the values of the dissociation constant ratio for the agonist at the two receptors. When the value of the dissociation constant for receptor II was greater than the value for receptor I, then the shape of the dose-response curve was determined by the interaction of the agonist with receptor I, the effect of the interaction with receptor II being fully masked. The effect of interaction with receptor II could be unmasked by means of a competitive antagonist for the interaction of the agonist with receptor I.

Our experimental findings fit remarkably well into these theoretical curves, derived by Ariëns *et al.* (1959) The dose-response curve for histamine appears to be determined by the interaction of histamine with the H_1-receptors and any effect resulting from interaction with the H_2-receptors is masked. This is consistent with the failure of metiamide, a competitive antagonist of histamine at the H_2-receptor, to modify the dose-response relation for histamine. The effects resulting from interaction with the histamine H_2-receptors were revealed by treatment with mepyramine, a competitive antagonist of histamine at the H_1-receptor In the hypothetical situation described by Ariens *et al.* (1959) the shape of the dose-response curve was determined by the interaction of the agonist with the receptor having the smaller dissociation constant, in our experiments the interaction of histamine with the H_1-receptors.

We are grateful to Mrs Helen Farrington, Mrs Ann Wonnacott and Mr Duncan Cameron for their valuable technical assistance.

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(Received January 13, 1975)

Br. J. Pharmac. (1975), 54, 325-332

THE EFFECTS OF TETRAPHENYLBORON ON NEUROMUSCULAR TRANSMISSION IN THE FROG

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1 The effects of tetraphenylboron (TPB) were studied on the frog sciatic nerve-sartorius muscle preparation.

2 TPB (0.01-1 mM) blocked indirectly elicited twitches of the preparation.

3 TPB (0.01-0.1 mM) produced no depolarization but lowered membrane resistance. TPB increased miniature endplate potential (m.e p.p.) frequency, the rate of rise of the endplate potential (e.p.p.), and slowed the rate of rise and rate of fall of the muscle action potential

4 In Mg^{2+} solutions the quantal content of e.p.ps was initially increased by TPB (0.01 mM). This was followed by a decrease of e.p.p. and m.e.p.p. amplitudes, accompanied by a lack of e.p.p. failures.

5 Larger concentrations of TPB (0.1 mM) produced an increase in e.p.p amplitude followed by the sudden abolition of e.p.ps. This effect was associated with abolition of the nerve terminal spike

6 TPB (0.1 mM) exhibited no postjunctional blocking action.

7 The results indicate that TPB acts prejunctionally, initially causing an increased release of acetylcholine. Subsequently, transmitter output is reduced by a reduction of quantal size.

Introduction

Tetraphenylboron (TPB), a lipid soluble anion first synthesized by Wittig, Keicher, Rückert & Raff (1949), has been employed in the gravimetric analysis of potassium salts (for review see Flaschka & Barnard, 1960) and of choline esters (Augustinsson & Grahn, 1954), and as a cell dispersing agent (Rappaport & Howze, 1966). It has also been suggested that TPB acts as a cholinoceptor agonist at both parasympathetic neuroeffector junctions (Guideri, Seifter & Seifter, 1968) and the neuromuscular junction (Seifter, Guideri & Seifter, 1968), by interacting with the esteratic site of the acetylcholine receptor (Guideri, Seifter & Seifter, 1972). The claim that cholinoceptor activation could be produced by combination with the esteratic site alone was not substantiated by Marshall (1970) who found that TPB produced no contracture of the chick biventer cervicis muscle. In addition Marshall (1970) postulated that the anti-curare activity of TPB (Seifter *et al.*, 1968) was due to an augmentation of evoked transmitter release rather than to a depolarizing action.

The experiments described have been designed to examine further the actions of TPB on neuromuscular transmission.

Methods

All experiments were performed on the frog (*Rana pipiens*) isolated sciatic nerve-sartorius muscle preparation. Nerve-muscle preparations were dissected in a phosphate-buffered Ringer solution (Parsons, 1969) and were subsequently maintained in a Tris (tris-(hydroxymethyl)aminomethane)-buffered Ringer solution (pH 7.25) at room temperature (17-20° C). The composition of the Tris-Ringer solution was (mM): NaCl 120, KCl 2 5, $CaCl_2$ 1.8, Tris 1. However, when TPB was added directly to the muscle chamber, KCl was omitted from the solution (K^+-deficient Ringer) to avoid precipitation of the water-insoluble potassium salt of TPB. Other modifications of these solutions are indicated in the text.

When necessary, neuromuscular block was produced by reducing the $CaCl_2$ concentration of the Tris-Ringer to 0.9 mM and adding 8 mM $MgCl_2$ Quantal content determinations were made in this solution by the ratio of e.p.p. to m.e.p.p. amplitudes (del Castillo & Katz, 1954).

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Statistical analyses were performed by Student's *t*-test, values of *P* less than 0 05 being regarded as significant. Values quoted in the text are expressed as mean ± standard error of the mean (s.e. mean).

Twitch tension experiments

In these experiments both sartorius nerve-muscle preparations were maintained in K^+-deficient Tris-Ringer solution. The sciatic nerve was stimulated at a frequency of 0.1 Hz by rectangular pulses of 50 μs duration, and of twice the strength required to produce a maximal muscle twitch. Isometric muscle twitches were recorded by Grass FTO3C strain gauges Nerve-muscle preparations were equilibrated for 30 min in K^+-deficient solution before addition of TPB to one of the preparations. The second preparation served as a control to monitor the effects of prolonged exposure to K^+-deficient solution.

Electrophysiological techniques

After dissection and mounting of the preparation, the entire muscle chamber was secured on the stage of a Bausch & Lomb dynoptic microscope. When transilluminated, it was possible to view the preparation under 300x magnification, using a Leitz long-range objective. By this technique, a nerve twig could be visualized down to the last node of Ranvier and a microelectrode inserted in the corresponding muscle fibre in the immediate vicinity of the chemosensitive post-junctional membrane.

Resting membrane potentials, effective membrane resistances, endplate potentials (e.p.ps), miniature endplate potentials (m.e.p.ps) and muscle action potentials were recorded intracellularly with micropipettes filled with 3 M KCl and having resistances in the range of 6-12 MΩ. Nerve terminal spike activity was recorded extracellularly with micropipettes filled with 1 M NaCl and of low resistance (1-2 MΩ). Both intracellular and extracellular signals were amplified by a solid state neutralized capacitance preamplifier (Industrial Science Associates, Ridgewood, N.Y) and were displayed simultaneously on Tektronix 565 & 564 oscilloscopes. Oscilloscope tracings were recorded either on Polaroid film or on moving film or paper by a Grass kymograph camera.

Endplate potentials, nerve terminal spikes and junctional action potentials were elicited by stimulation of the sciatic nerve through bipolar platinum electrodes with rectangular pulses of 50 μs duration and of sufficient strength to produce a maximum response. Directly elicited muscle action potentials were produced by stimulating an individual muscle fibre through a second microelectrode inserted in the same fibre approximately 1.3 mm from the recording electrode, with rectangular pulses of 0.01-0.05 ms duration and of sufficient strength to elicit an action potential.

Drug application techniques

TPB was either added to the solution bathing the whole muscle, or perfused directly onto the endplate region of individual muscle fibres (Manthey, 1966; Johnson & Parsons, 1972). In the former case the muscle was bathed in K^+-deficient Tris-Ringer solution, and the responses from many different fibres were measured during exposure to TPB. In the latter case the effect of TPB on the responses of individual fibres was measured over a prolonged period. In this case the muscle was bathed in Tris-Ringer solution although TPB was dissolved in K^+-deficient Tris-Ringer solution. Except for potassium and TPB the perfusion solutions always contained the same constituents as the bathing solution. Microperfusion of individual endplates was achieved by rapidly lowering a micropipette (50-100μm tip diameter) containing the perfusing solution into the bathing medium to a position about 0.1 mm above the fibre surface. The perfusion solution was delivered by hydrostatic pressure from a 15 cm column of solution.

Results

Effects of tetraphenylboron added to the bath

Effects on twitch tension development in indirectly stimulated muscles Relatively large concentrations of TPB (0.1 mM and over) were required to produce measurable effects on twitch tension. TPB 1 mM produced an immediate reduction in twitch amplitude, complete block being obtained in 1-4.5 min (mean 2.5 min; five experiments). The blocking effect of TPB 0.5 mM was slightly slower, taking 3-7 min (mean 5 min; four experiments) to achieve complete inhibition. At both 1 mM and 0.5 mM the blockade of twitch tension could occasionally be relieved by washing, especially if TPB was removed immediately after abolition of twitch tension. However, if the preparation was exposed to TPB (0.5 or 1 mM) for 5-10 min beyond the time of complete block, twitch tension did not recover even with prolonged washing

The blocking effects of TPB 0.1 mM were slow in onset requiring 12-55 min (mean 34 min; four

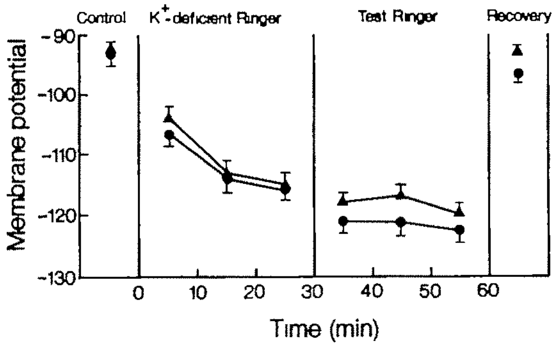

Figure 1 The effects of K^+-deficient solution and subsequent treatment with tetraphenylboron (TPB) 0.1 mM on resting membrane potential. Control values represent the mean (± s.e. mean) of 15-25 potential measurements from individual fibres after approximately 30 min equilibration in normal Tris-Ringer solution. All seven muscles used were then exposed to K^+-deficient solution for 30 minutes. After this period four muscles were exposed to K^+-deficient Ringer plus TPB 0.1 mM (▲), whereas the other three muscles remained in K^+-deficient solution (●). All points represent the mean of measurements made in 6-25 individual fibres. Vertical lines show s.e. mean. Recovery values represent the mean (± s.e. mean) of measurements made in 17 individual fibres 10-30 min after returning the muscles to normal Tris-Ringer solution.

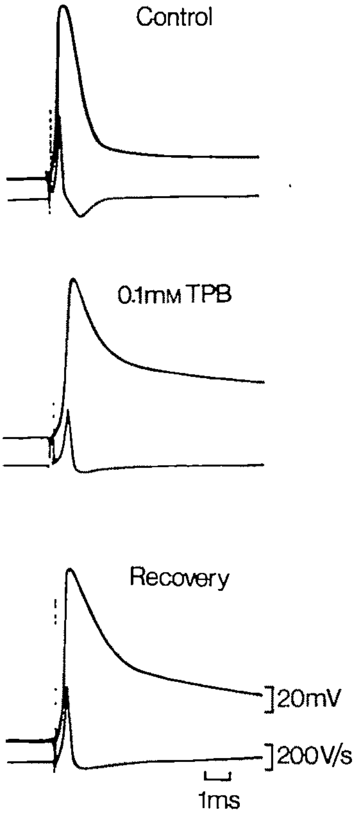

Figure 2 The effects of tetraphenylboron (TPB) 0.1 mM on directly elicited muscle action potentials. The figure shows results obtained in three different fibres in the same muscle. In each case the upper trace is the intracellularly recorded transmembrane potential (Vm), and the lower trace is dVm/dt obtained by electronic differentiation. Traces have been retouched slightly.

experiments) for complete blockade, and were generally preceded by a period of slight (up to 12%) twitch augmentation. The blocking effects of TPB 0.1 mM could not be reversed even by extensive washing.

Concentrations of TPB lower than 0.1 mM produced no change in twitch tension development in preparations exposed to TPB for periods of up to one hour.

Effects on some electrical properties of the muscle fibre Membrane potentials were measured initially in three muscles equilibrated for at least 30 min in the Tris-Ringer solution. The Tris-Ringer solution was then replaced by K^+-deficient Tris-Ringer solution and the membrane potential of different fibres sampled over a period of 60 minutes. In the K^+-deficient solution a rapid hyperpolarization followed by a gradually-developing further hyperpolarization was evident (Figure 1). Such a hyperpolarization has also been observed in K^+-deficient solution by Akiyama & Grundfest (1971). When the muscle was returned to the Tris-Ringer solution the resting potentials fell to control levels. In a comparable series of experiments, in four muscles after 30 min exposure to K^+-deficient solution, the bathing solution was changed to one containing TPB 0.1 mM. The hyperpolarizations observed during 30 min of TPB treatment were not significantly different from those observed during the last 30 min of exposure to K^+-deficient solution (Figure 1).

The influence of TPB 0.1 mM on the input resistance of individual muscle fibres was determined in four preparations. The input resistance measured in muscle fibres equilibrated for at least 15 min in K^+-deficient solution was 0.91 ± 0.06 x $10^6\Omega$ ($n = 29$). Subsequent exposure to TPB 0.1 mM for at least 15 min reduced the input resistance significantly ($P < 0.05$) to 0.54 ± 0.03 x $10^6\Omega$ ($n = 27$).

TPB altered the configuration of directly stimulated muscle action potentials. Action poten-

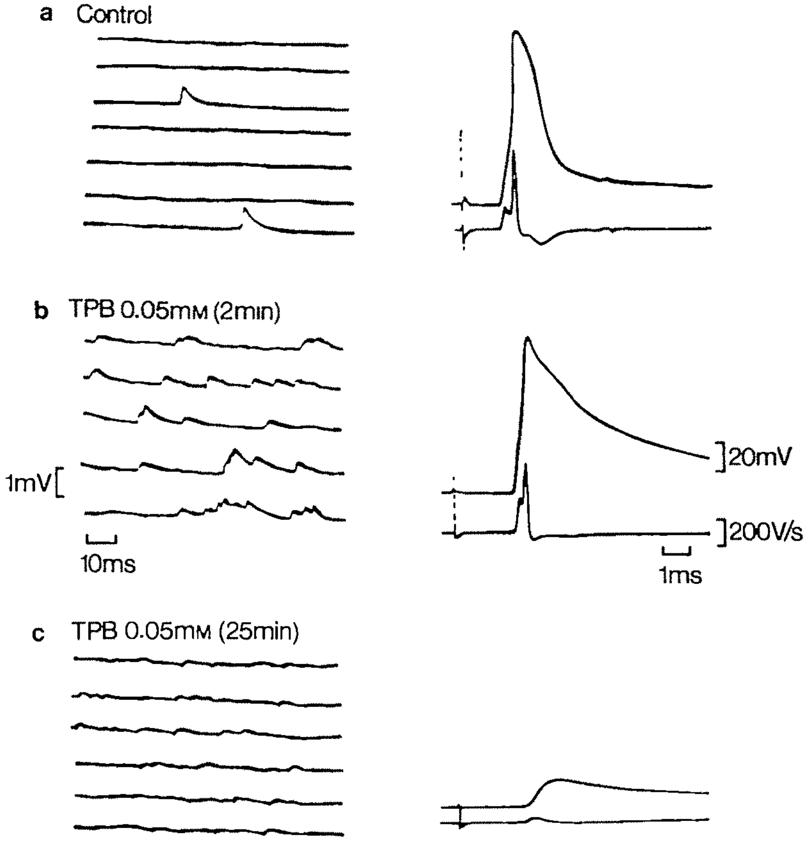

Figure 3 The effects of tetraphenylboron (TPB) 0.05 mM on miniature endplate potentials (m e.p ps), endplate potentials (e.p ps) and indirectly elicited muscle action potentials The figure shows results obtained in three different fibres of the same muscle. In each fibre representative records of m.e.p ps (left panels) were obtained prior to recording the effects of nerve stimulation (right panels) In the right panels the upper trace is the transmembrane potential and the lower trace is dVm/dt as in Figure 2. Traces have been retouched slightly Note that the first action of TPB is to increase dVm/dt of the e.p.p. (b) whereas subsequently the e.p.p becomes subthreshold (c)

tials were recorded first in muscle fibres equilibrated in K^+-deficient Ringer solution, and then from fibres equilibrated for 10-35 min in the K^+-deficient, TPB 0.1 mM solution TPB depressed the maximum rate of rise, the maximum rate of fall and the overshoot and greatly prolonged the duration of the action potential (Figure 2). For example, in the muscle preparation illustrated in Figure 2 the maximum rate of rise of the action potential was decreased from 604 ± 6 V/s (mean ± s.e mean, n =5) to [illegible]0 ± 15 V/s, the maximum rate of fall was decreased from 148 ± 2 V/s to 59 ± 1 V/s and the overshoot was reduced from 42 ± 1 mV to 25 ± 1 mV following exposure to TPB 0.1 mM. These changes were only partially reversed after prolonged washing (15-70 min) with TPB-free solution For example, in the preparation illustrated in Figure 2 the maximum rate of rise of the action potential recovered to 540 ± 19 V/s, the maximal rate of fall to 61 ± 0.5 V/s and the overshoot to 30 ± 1 mV (n = 5) Similar changes were observed in four muscles

Effects at the endplate region of individual muscle fibres TPB exhibited concentration- and time-dependent effects at individual motor endplates both on the electrical activity following nerve stimulation, and on miniature endplate potential activity These effects were observed at a lower concentration range (0.01-0.1 mM) than those used in the twitch tension experiments. TPB, in concentrations up to 0 1 mM produced no endplate depolarization

In the presence of TPB 0.01, 0.05 and 0.1 mM the maximum rate of rise of the e.p.p. was consistently increased, and the time course of the junctional action potential, like that of the directly-elicited action potential, was prolonged (Figure 3). Concomitantly, there was a large increase in m.e.p.p. frequency above control levels (1-2/s), ranging from 40/s (0.01 mM TPB) to over 300/s (0.1 mM TPB). During 60 min exposure to TPB 0.01 mM the changes in e.p.p. and junctional action potential were maintained and were not reversed by 30 min in TPB-free solution.

In the presence of TPB 0.05 or 0.1 mM neuromuscular block of surface fibres occurred, although the pattern of blockade produced by the two concentrations differed.

In the presence of TPB 0.05 mM (three different nerve-muscle preparations) a gradual blockade of neuromuscular transmission developed, as indicated by the appearance of subthreshold e.p.ps after 5, 8 and 17 min exposure respectively (Figure 3). As the time of exposure to TPB 0.05 mM was increased (up to 40 min) the amplitude of the subthreshold e.p.p. continued to decline, in parallel with a decline in the m.e.p.p. amplitude. Frequency of m.e.p.ps also declined from a peak of over 100/s to around 20-60/s. After washing, the m.e.p.p. and e.p.p. amplitudes increased in all three preparations and in one preparation full transmission was restored. Frequency of m.e.p.ps remained elevated (10-100/s) over a 30-60 min recovery period after washing.

In the presence of TPB 0.1 mM complete neuromuscular block occurred precipitously within 5-10 min (three preparations). At this time m.e.p.ps, although reduced in amplitude, were still obvious. Continued exposure to TPB led to further reduction of m.e.p.p. amplitude and frequency. After washing subthreshold e.p.ps were observed in a few fibres of only one preparation. Nevertheless, m.e.p.p. frequency was considerably greater than control, and m.e.p.p. amplitude progressively approached control levels.

Effect on postjunctional sensitivity to carbachol The depolarization of the postjunctional membrane produced by micro-perfusion of carbachol 0.014 mM at individual endplates was measured in fibres equilibrated in K^+-deficient solution for at least 30 min, and in different fibres of the same muscle, after exposure to K^+-deficient TPB 0.1 mM solution for at least 30 minutes. At this time no m.e.p.ps were observed in most TPB-treated fibres. In all cases the depolarization produced by carbachol (0.014 mM) was greater in the TPB-treated fibres, the mean depolarization of 28.5 ± 4.1 mV (mean ± s.e. mean) in the four control fibres being significantly less than that (51.7 ± 4.5 mV) in the four TPB-treated fibres ($P < 0.05$).

Effects of microperfused tetraphenylboron

Effects on endplate potentials To study more quantitatively the relationship between the effects of TPB and time of exposure in individual fibres, the drug was locally microperfused onto individual junctional regions, partially blocked by Mg^{2+} to prevent muscle twitching.

Quantitative determinations were made by comparing the amplitude of e.p.ps and m.e.p.ps during a control perfusion of K^+-deficient Ringer solution with those during perfusion of TPB. With this method, TPB 0.1 mM produced a large increase in e.p.p. amplitude and m.e.p.p. frequency, often accompanied by muscle spiking and contractions, followed rapidly by the precipitous loss of the e.p.p. response. At this time m.e.p.p. frequency remained high. The lower concentration of 0.01 mM TPB produced no sudden fall in amplitude and hence quantal content determinations were performed at this concentration. The increase in mean quantal content ranged from 3.93-14.45 times control in five fibres (Table 1). During control perfusion of K^+-deficient solutions many e.p.p. failures were observed; however, during perfusion with TPB 0.01 mM no e.p.p. failures were evident.

Table 1 Initial effects of tetraphenylboron (TPB, 0.01 mM) on mean quantal content of endplate potentials recorded in Tris-Ringer solution containing $CaCl_2$ (0.9 mM) and $MgCl_2$ (8 mM)

Fibre no.	*Control*			*TPB-treated (5 min)*			
	m.e.p.p. amplitude (mV)	*e.p.p. amplitude (mV)*	*mean quantal content (m)*	*m.e.p.p. amplitude (mV)*	*e.p.p. amplitude (mV)*	*mean quantal content* $(\bar{m})$	$\frac{m(TPB)}{m(control)}$
1	0.39	0.41	1.05	0.35	4.34	12.39	11.80
2	0.53	1.70	3.19	0.47	14.95	32.03	10.04
3	0.60	0.90	1.50	0.62	5.36	8.59	5.72
4	0.64	0.35	0.55	0.46	3.62	7.92	14.45
5	0.43	0.86	1.99	0.38	2.97	7.82	3.93

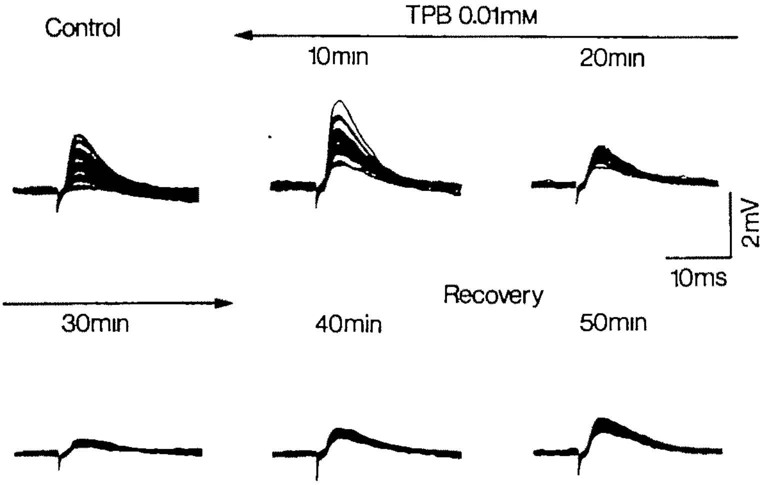

Figure 4 The effects of tetraphenylboron (TPB) 0.01 mM on endplate potentials (e.p ps) recorded in 0.9 mM $CaCl_2$. 8 mM $MgCl_2$ Tris-Ringer solution. In these experiments all results are from a single fibre monitored for one hour. TPB was applied to the endplate region by microperfusion for 80 min followed by local perfusion with Tris-Ringer solution during the recovery period. Each panel shows 20 superimposed e.p.ps Note the presence of several failures and wide spread of amplitudes during control measurements. In contrast, after 30 min exposure to TPB no failures are evident.

To study the effects of prolonged exposure to TPB 0.01 mM the membrane potential was maintained at −80 mV in three fibres by passing, if necessary, a d.c. current into the endplate region via a second microelectrode inserted within 50 μm of the recording electrode. Under these conditions TPB (0.01 mM) produced an initial increase in e.p.p. amplitude and m.e.p.p. frequency, followed by a gradual decrease in e.p.p. amplitude that paralleled a fall in m.e.p.p. frequency from its previously high level. No change in the time course of the e.p.p. was noted. The progressive decrease in e.p.p. amplitude was not accompanied by the appearance of failures (Figure 4). The decrease in e.p.p. amplitude was partially reversible by washing. Mean quantal content determinations were performed in two fibres during prolonged microperfusion of TPB (0.01 mM). Initially mean quantal content was increased (3.4 and 2.7 times control respectively), and as e.p.p. amplitude fell after approximately 45 min exposure to TPB, remained greatly above control (4.4 and 2.6 times control respectively). As the final determinations were made at a time when m.e.p.p. amplitude was approaching the noise level of the fibres, these latter determinations are probably underestimates of mean quantal content as only those m.e.p.ps with amplitudes greater than the noise could be measured.

Microperfused TPB produced no reduction of postjunctional sensitivity. In three fibres the depolarization produced by carbachol 0.014 mM (15-18 mV) after 10-15 min local perfusion of TPB (0.1 mM) was as large as that produced by control perfusions of carbachol (14-16 mV).

As TPB had been shown to be capable of augmenting transmission under conditions of low release i.e. low calcium, high magnesium concentrations, it was of interest to determine whether TPB could restore transmission in the absence of calcium. Accordingly muscles were equilibrated in Tris-buffer solutions containing no $CaCl_2$ and 1 mM EGTA ([Ethylenebis(oxyethylenenitrilo)]-tetraacetic acid), and either 2 mM or 4 mM $MgCl_2$. Under these conditions no e.p.ps were elicited by nerve stimulation, and none were observed in the presence of 0.01 or 0.05 mM TPB (three determinations at each concentration) although m.e.p.p. frequency was greatly increased.

Effects on nerve terminal spike activity As the precipitous abolition of e.p.ps observed in the presence of high concentrations was suggestive of a depression of nerve terminal function, extracellular recording of nerve terminal spike (NTS) activity and extracellular e.p.ps were made in the 0.9 mM $CaCl_2$ and 8 mM $MgCl_2$ Tris-Ringer solution. Perfusion of K^+-deficient Ringer solution produced no effect on either NTS or e.p.p activity. Perfusion of TPB 0.1 mM occasionally produced such a large initial increase in e.p.p. amplitude that action potentials and twitching were observed. However, in most preparations the initial rise in e.p.p. amplitude was rapidly followed by the simultaneous abolition of NTS and e.p.p. Both the NTS and e.p.ps returned when TPB was removed. No attempt was made to quantitate changes in configuration of the NTS by concentrations of TPB less than 0.1 mM.

Discussion

In the present study no depolarization of the postjunctional membrane was noted when TPB was applied either by addition to the bath or by microperfusion. The observation does not support the view that the negatively charged TPB can activate acetylcholine receptors (Seifter *et al.*, 1968). The results show rather that TPB possesses potent activity on transmitter release, both spontaneous and evoked, and on the ionic conductance properties of the electrically excitable membranes.

The effects of TPB on transmitter release were both concentration- and time-dependent. The initial effect of TPB was to produce an increase both in m.e.p.p. frequency and e.p.p. quantal content. Both of these phenomena may be related to the ability of the lipid-soluble TPB to penetrate the nerve terminal and inhibit calcium uptake by mitochondria, an action it is known to possess in liver cells (Utsumi & Packer, 1967). The possible importance of mitochondria in regulating intraneuronal calcium levels has recently been discussed by Alnaes, Meiri, Rahamimoff & Rahamimoff (1974). Increased intracellular calcium concentration is associated with increases both in m.e.p.p. frequency (Miledi & Thies, 1971) and in e.p.p. quantal content (Crawford & Fettiplace, 1971). It is thought that tetraethylammonium (TEA) increases quantal content by prolonging the presynaptic spike, by a similar mechanism to that by which it prolongs the muscle action potential, namely a decrease in activated potassium conductance (Benoit & Mambrini, 1970). Like TEA, TPB prolongs the muscle action potential, and it is conceivable that concentrations of TPB lower than 0.1 mM may increase the quantal content in part by a prolongation of the nerve terminal spike. Nevertheless, it should be noted that TPB is different from TEA in that the latter drug does not increase m.e.p.p. frequency (Benoit & Mambrini, 1970).

After the initial increase in quantal content, evoked neuromuscular transmission was blocked by two mechanisms. At 0.1 mM, the sudden elimination of the e.p.p. or junctional action potential was shown to be due to an abolition of the nerve terminal spike. As no depolarization was noted in either junctional or non-junctional areas of the muscle fibre, it is unlikely that the nerve terminal block was due to terminal depolarization. However, TPB slowed the rate of rise of muscle action potentials, an effect indicative of a reduced sodium content. Such an action in the fine nerve terminals could lead to nerve terminal block. With prolonged application of lower concentrations (0.01-0.05 mM) of TPB there was a gradual decrease of e.p.p. amplitude, in parallel with a decrease of m.e.p.p. amplitude and frequency, associated with a lack of e.p.p. failures. The lack of failures in the presence of TPB indicates that the probability of transmitter release was above the pre-TPB level. Consequently the decrease in e.p.p. amplitude cannot be attributed to a slowly-developing inhibition of the release process. In addition, as mean quantal content remained well above control levels after continued exposure to TPB the fall in e.p.p. amplitude is probably due either to a fall in quantal size, or to a postjunctional blocking action of TPB.

TPB (0.1 mM) added to the bath increased the depolarization by carbachol (0.014 mM). However, no similar sensitization was observed when TPB was locally applied for only 8-12 min prior to carbachol application. The enhancement produced by TPB added to the bath also occurred in fibres voltage-clamped at −100 mV (Parsons & Spannbauer, unpublished observation), and thus could not be due to either an increase in driving force caused by the slowly developing hyperpolarization (Figure 1) or to an increase in membrane resistance. A similar time-dependent sensitization by 1-fluoro-2,4-dinitrobenzene has recently been described by Edelson & Nastuk (1973).

In view of the lack of postjunctional blocking action of TPB the reduction of e.p.p. amplitude is probably due to a reduction in quantal size, as indicated by the fall in m.e.p.p. amplitude.

This work was supported by NIH Grant NS-07740 to R.L.P. and was done during the tenure of a Research Fellowship of Muscular Dystrophy Associations of America, and a Wellcome Research Travel Grant to I.G.M. We also acknowledge the valuable assistance of Mr P Spannbauer. Please send reprint requests to Dr R.L. Parsons in Vermont.

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(Received December 12, 1974.
Revised March 11, 1975.)

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THE EFFECTS OF TETRAPHENYLBORON ON SPONTANEOUS TRANSMITTER RELEASE AT THE FROG NEUROMUSCULAR JUNCTION

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1 The effects of tetraphenylboron (TPB) on spontaneous transmitter release were studied in the frog sartorius muscle preparation.

2 TPB (0.001-1 mM) produced a time-dependent increase in miniature endplate potential (m.e.p.p.) activity that was not sustained. TPB (0.1 mM) produced similar effects on m.e.p.p. frequency in normal Ringer solutions, in the absence of Ca^{2+} or Cl^- and in the presence of excess Ca^{2+} and of tetrodotoxin. The effect of TPB (0.01 mM) was reduced but not abolished in the absence of Ca^{2+}.

3 As m.e.p.p. frequency fell from its peak level in TPB (0.04 mM) m.e.p.p. amplitude was reduced. The reduction of m.e.p.p. amplitude was not prevented by choline (30-300 μM).

4 When m.e.p.p. activity fell below the noise level in the presence of TPB (0.1 mM), lanthanum (0.5 mM) was ineffective in promoting measurable m.e.p.p. activity.

5 The effects of TPB were slowly reversible by washing.

6 The results indicate that TPB acts to reduce the nerve terminal stores of acetylcholine, probably by a combination of rapid release and concomitant inhibition of transmitter storage.

Introduction

Amongst the most striking effects of tetraphenylboron described in the previous paper (Marshall & Parsons, 1975) were a marked increase in spontaneous transmitter release, and a subsequent time-dependent decrease in miniature endplate potential (m.e.p.p.) amplitude and frequency. The present study represents attempts to determine the mechanisms responsible for the changes in m.e.p.ps produced by this negatively charged compound. Some of these results have been presented to the American Society for Pharmacology and Experimental Therapeutics at Montreal (Marshall & Parsons, 1974).

Methods

Conventional intracellular recording techniques, as described in the previous paper (Marshall & Parsons, 1975), were used to record miniature endplate potentials (m.e.p.ps) from the frog sartorius muscle. TPB, dissolved in K^+-deficient Ringer solution (Marshall & Parsons, 1975), was applied to individual neuromuscular junctions by local microperfusion. Muscles were bathed in Tris-buffered Ringer solution (composition (mM) NaCl 120, KCl 2.5, $CaCl_2$ 1.8, Tris 1) except when otherwise stated. Except for TPB and KCl the perfusion solutions always contained the same constituents as the bathing solution. Frequency and amplitude of m.e.p.ps were measured from photographic records of oscilloscope traces.

Statistical analyses were performed by Student's *t*-test, values of *P* less than 0.05 being regarded as significant. Values quoted in the text are expressed as mean ± standard error of the mean (s.e. mean).

Results

Effects on m.e.p.p. frequency

Microperfusion of TPB onto individual surface junctional regions produced an increase in m.e.p.p. frequency within a few seconds. After the attainment of peak frequency, there was a gradual fall of frequency. In addition, as the m.e.p.p. frequency decreased, m.e.p.p. amplitude was progressively reduced. Consequently, the accurate measurement

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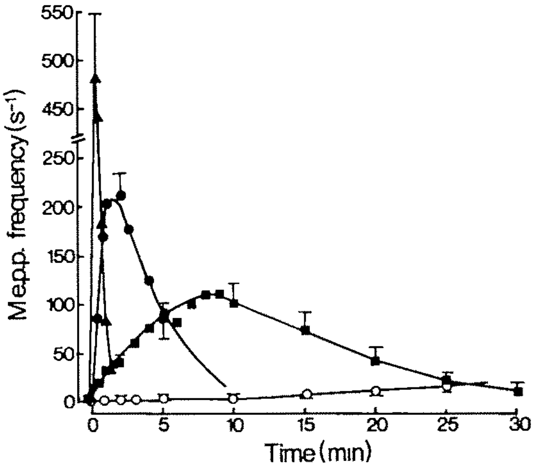

Figure 1 Effects of tetraphenylboron (TPB) on miniature endplate potential (m e p.p.) frequency in Tris-Ringer solution. The relationships between time of microperfusion and m.e.p.p frequency produced by 1 mM (▲), 0.1 mM (●), 0.01 mM (■) and 0.001 mM (○) TPB are shown. Each point represents the mean of 6 determinations. Vertical lines show s.e mean.

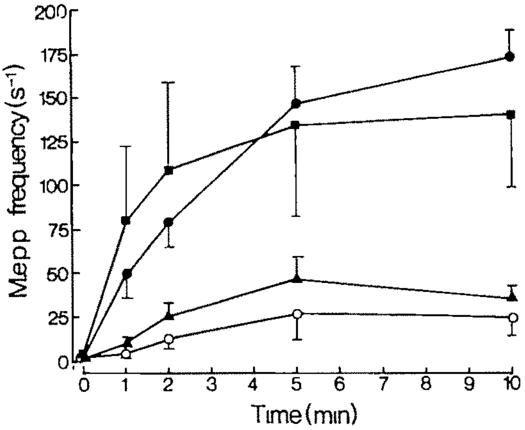

Figure 2 Effects of tetraphenylboron (TPB) (0.01 mM) on miniature endplate potential frequency in normal Tris-Ringer solution (1.8 mM $CaCl_2$, 0 mM $MgCl_2$; ●), high Ca^{2+} Tris-Ringer solution (10 mM $CaCl_2$, 0 mM $MgCl_2$; ■), Ca^{2+}-deficient Tris-Ringer solutions (0 mM $CaCl_2$; 8 mM $MgCl_2$, 1 mM EGTA; ▲, 0 mM $CaCl_2$; 2 mM $MgCl_2$; 1 mM EGTA ○). Each point represents the mean of 5 determinations. Vertical lines show s.e. mean.

of m.e.p.p. frequency was limited as the m.e.p.p. amplitude approached the base-line noise (approx 0.1 mV).

The effect of TPB was both time- and concentration-dependent (Figure 1). Thus TPB 1 mM produced an explosive increase in m.e p.p. frequency, followed within 2-3 min by the disappearance of m.e.p.p. activity. In contrast, TPB 0.01 mM produced a slower and less marked increase followed by a loss of measurable activity in approximately 30 minutes. After abolition of m.e.p.p. activity by TPB 0.1 mM, perfusions of either TPB 1 mM or lanthanum 0 5 mM, concentrations that in untreated fibres produced large increases in m.e.p.p. frequency, did not produce any measurable m.e.p.p. activity. It should be noted that at the higher concentrations of TPB the m.e.p.p. frequency was so high that there was summation of individual m e.p.ps and the accuracy of measurement of frequency from photographic records was limited at frequencies of over 200-300/second. Therefore, the mean values shown on graphs should be regarded as estimates.

Control perfusions of K^+-deficient Tris-Ringer solution produced no significant effects on m.e.p.p. frequency (Table 1).

In order to determine the mechanism of action of TPB on m e.p.p. frequency, several possible factors modifying the action of TPB were studied. The effects of TPB 0.1 mM on m.e.p.p.

Table 1 Effects of Ca^{2+} concentration on the increase in m.e.p.p. frequency produced by tetraphenylboron (TPB) 0.1 mM.

Perfusion solution	*Bathing solution*			*M.e.p.p. frequency/s*			
		Control	*1 min*	*2 min*	*5 min*	*10 min*	n
K^+-deficient Tris	Tris	0.4 ± 0.1	0.2 ± 0.1	0.3 ± 0.2	0.6 ± 0.2	1 ± 0.6	6
0.1 mM TPB	Tris	2.2 ± 0.5	203 ± 30	210 ± 26	91 ± 11	10 ± 7	6
0.1 mM TPB	0 mM Ca^{2+} 8 mM Mg^{2+}	4.4 ± 2.6	218 ± 89	198 ± 70	154 ± 30	61 ± 29	6
0.1 mM TPB	0 mM Ca^{2+} 8 mM Mg^{2+} 1 mM EGTA	1.3 ± 0.6	328 ± 13	249 ± 33	54 ± 50	N.M.	3
0.1 mM TPB	10 mM Ca^{2+}	1.1 ± 0.4	236 ± 60	159 ± 31	57 ± 46	12 ± 12	4

N.M. Not measured

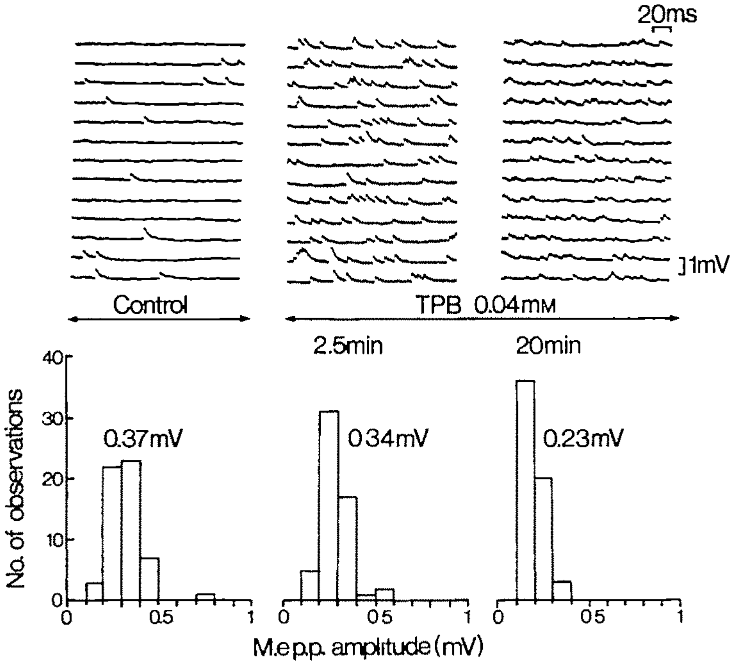

Figure 3 Effects of tetraphenylboron (TPB) 0.04 mM on miniature endplate potential (m.e.p.p.) frequency and amplitude in 10% hypertonic Tris-Ringer solution. The upper panels illustrate the effects of TPB on intracellularly recorded m.e.p.ps. The lower panels are the amplitude distributions corresponding to the records shown; mean m.e.p.p. amplitudes are inscribed. All records are from the same fibre.

frequency in Tris-Ringer solutions were not significantly different ($P > 0.05$) from its effects in Ca^{2+}-deficient Tris-Ringer solutions containing $MgCl_2$ 8 mM and from those in solutions containing $CaCl_2$ 10 mM (Table 1). A marked increase in frequency was also obtained in 3 fibres maintained in Ca^{2+}-deficient solutions containing EGTA 1 mM ([Ethylenebis(oxyethylenenitrilo)]-tetraacetic acid; Table 1).

In these experiments the increase in m.e.p.p. frequency produced by TPB 0.1 mM was so great that any small differences in frequency would have been difficult to detect. Thus, additional experiments were conducted with TPB 0.01 mM. At this concentration $CaCl_2$ 10 mM did not significantly increase m.e.p.p. frequency above that recorded in $CaCl_2$ 1.8 mM, whereas in solutions without $CaCl_2$ containing EGTA 1 mM and either 2 mM or 8 mM $MgCl_2$ the increase in m.e.p.p. frequency was significantly reduced, but was not abolished (Figure 2).

TPB (0.1 mM) also produced similar effects on m.e.p.p. frequency to those seen in Tris-Ringer solution, in Cl^--deficient solutions (replacing chloride salts by nitrate salts) and in Tris-Ringer solutions containing tetrodotoxin 1 ng/ml.

Effects on m.e.p.p. amplitude

In the experiments in which changes in m.e.p.p. amplitude were quantified, TPB 0.04 mM was used. At this concentration the change in m.e.p.p. frequency developed slowly, thus allowing accurate measurements of m.e.p.p. amplitude. However, even at this concentration m.e.p.p. activity became indistinguishable from noise within 20-30 minutes. To increase the number of control m.e.p.ps, the resting m.e.p.p. frequency was raised by elevating the tonicity of the Ringer solution 10% by the addition of 25 mM sucrose. In addition, the membrane potential was maintained at −80 mV by passing d.c. current through a second intracellular electrode.

During the initial moments of TPB application, as m.e.p.p. frequency rose, no marked effect on m.e.p.p. amplitude was observed. Measurement of m.e.p.p. amplitude was not attempted at the peak m.e.p.p. frequency in TPB because of summation of m.e.p.ps. As frequency fell it was possible to measure individual m.e.p.ps. At this time TPB produced a large reduction of m.e.p.p. amplitude. The results obtained in one fibre are shown in Figure 3. In many fibres a few m.e.p.ps of large

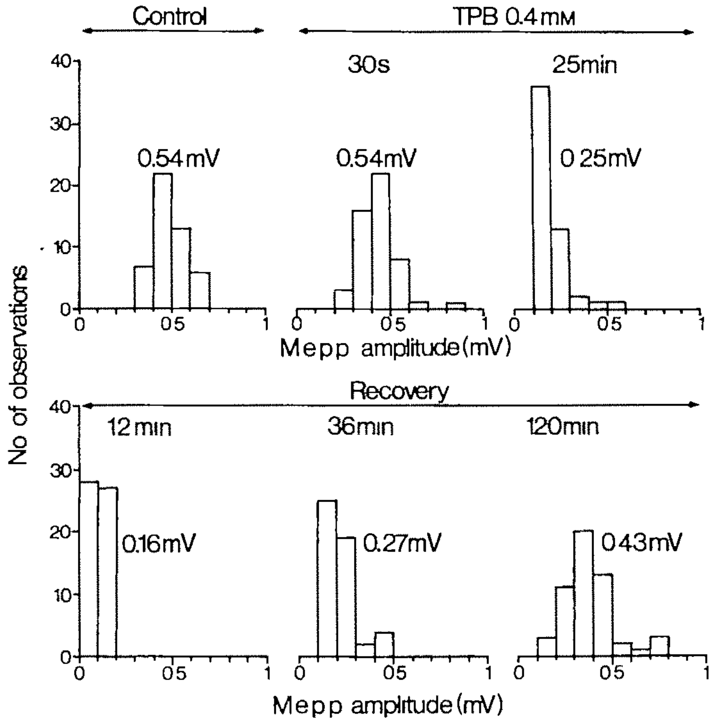

Figure 4 Reversibility of tetraphenylboron (TPB) action on miniature endplate potential (m e p.p.) amplitude The panels represent amplitude distributions, and mean m.e.p.p. amplitudes, measured in 10% hypertonic Tris-Ringer solution. All distributions are from the same fibre. TPB 0 04 mM was microperfused for 60 min at which time no m.e.p.p. activity had been detectable for 10 minutes Two amplitude distributions taken during the perfusion are shown in the upper panels. Perfusion was then continued with 10% hypertonic Tris-Ringer solution for a further 120 min and m.e.p.p amplitude distributions made during recovery of amplitude (lower panels).

amplitude (0.5-1.5 mV) were observed at a time when m.e.p.p. activity was almost indistinguishable from noise.

Reversibility of tetraphenylboron effects

In preliminary experiments it was observed that removal of TPB (0.1 mM) from the muscle chamber after abolition of m.e p.p. activity resulted in the reappearance of m.e.p.ps. Initially the m.e.p.ps were of low amplitude and high frequency and, over a period of 2 h, as amplitude recovered towards control levels, frequency fell towards control. In two fibres perfusions of lanthanum (0.5 mM) during this recovery period produced progressively greater stimulating effects on m.e.p.p. frequency as recovery proceeded.

Amplitude distributions during the recovery of m.e.p.p. amplitude and frequency from the effects of TPB 0.04 mM were constructed in 3 fibres. As recovery was measured in individual fibres, washing was performed by replacing the TPB-containing perfusion pipette by one containing normal Tris-Ringer solution. The example in Figure 4 shows that m.e.p.p. amplitude recovered to 80% of control after 2 h of perfusion with normal Tris-Ringer solution. During the recovery phase, exceptionally large m.e.p.ps (1-1.5 mV) and bursts of m.e.p.ps were occasionally observed in several fibres

Effect of choline on the action of tetraphenylboron

To determine if the reduction in m.e.p.p. amplitude produced by TPB was a consequence of a hemicholinium-like inhibitory action on neuronal choline uptake, experiments were repeated in which TPB 0.1 mM was perfused onto neuromuscular junctions bathed in choline (30 μM −2 fibres and 300 μM −4 fibres) plus glucose (11 mM). Under these conditions TPB produced a rise in m.e.p.p. frequency followed by a fall in both m.e.p.p. frequency and amplitude. These effects were indistinguishable from the effects of control perfusions of TPB 0.1 mM

Discussion

The main effects of TPB on spontaneous transmitter release were an initial increase in m.e.p.p activity followed by the abolition of such activity. The dramatic increase in spontaneous acetylcholine release was similar to that produced by black widow spider venom (Longenecker, Hurlbut, Mauro & Clark, 1970) and by lanthanum (Blioch, Glagoleva, Liberman & Nenashev, 1968, De Bassio, Schnitzler & Parsons, 1971), being concentration-dependent, and being produced in the absence of extracellular Ca^{2+}. That a large concentration of TPB (0.1 mM) increased m.e.p.p. frequency approximately equally in the presence of and in the absence of Ca^{2+}, indicates that the effect of this concentration is not dependent upon entry into the nerve terminal of extracellular Ca^{2+}. The effect of this large concentration of TPB differed from that of lanthanum in that lanthanum is less effective in the presence of excess Ca^{2+} levels (De Bassio *et al.*, 1971) However, the action of the lower concentration of TPB tested (0.01 mM) was shown to be reduced but not abolished in the absence of extracellular Ca^{2+} indicating that, at low concentrations of TPB, Ca^{2+} entry into the nerve terminals contributes in part to the observed increase in m.e.p.p. frequency. In addition, the action of TPB is independent of chloride ions and unlike that of batrachotoxin (Jansson, Albuquerque & Daly, 1974) and of tityustoxin (Warnick, Albuquerque & Diniz, 1974) is not antagonized by tetrodotoxin.

Currently it is believed that the rate of spontaneous transmitter release is related to intraneuronal Ca^{2+} levels (Miledi & Thies, 1971) Further it is believed that intraneuronal Ca^{2+} levels are regulated to a great extent by mitochondrial sequestration (Alnaes, Meiri, Rahamimoff & Rahamimoff, 1974). Agents such as β-bungarotoxin (Wagner, Mart & Kelly, 1974), ruthenium red, and dicoumarol (Rahamimoff & Alnaes, 1973) and 1-fluoro-2,4-dinitrobenzene (Edelson & Nastuk, 1973) that inhibit mitochondrial Ca^{2+} accumulation or oxidative phosphorylation are known to increase m.e.p.p. frequency. A similar mechanism of action may be responsible for the effects of TPB on m.e.p.p. frequency, as this compound is known to inhibit both mitochondrial Ca^{2+} uptake and oxidative phosphorylation (Utsumi & Packer, 1967). Ready penetration of the highly-lipid soluble TPB into the nerve terminal may account for its rapid onset of action (Liberman & Topaly, 1968), particularly at the higher concentrations used where the action of TPB appeared independent of extracellular Ca^{2+} concentrations.

In TPB, as in black widow spider venom (Longenecker *et al.*, 1970) and lanthanum (De Bassio *et al.*, 1971), the peak m.e.p.p. frequency was not maintained. In the case of TPB this drop in frequency was accompanied by a reduction in m.e.p.p. amplitude which was not due to a post-junctional blocking action (Marshall & Parsons, 1975). In contrast, after a similar period of perfusion of lanthanum 0.5 mM we observed that m e.p.p. frequency stabilized at 30-100/s, without such a marked fall in amplitude. With black widow spider venom the fall in m.e.p.p. frequency to below control levels, is not associated with a consistent fall in m.e.p.p. amplitude (Longenecker *et al.*, 1970). Thus the fall in m.e.p.p. amplitude produced by TPB does not appear to be simply a consequence of the rapid rate of release. However, the rapid rate of release in combination with an inhibitory action of TPB on either the synthesis or the storage of acetylcholine might be expected to reduce m.e.p.p. amplitude. For example hemicholinium with repetitive nerve stimulation produces a reduction of quantal size (Elmqvist & Quastel, 1965, Jones & Kwanbunbumpen, 1970). However, TPB does not inhibit choline acetyltransferase *in vitro* (Guiden, Seifter & Seifter, 1972) and the lack of antagonistic action of choline suggests that a competitive inhibition of choline uptake is unlikely. An alternative mechanism of action of TPB is an inhibition of vesicular storage of acetylcholine. Recently, it has been proposed that type A botulinum toxin, which reduces m.e.p.p. amplitude possesses such an inhibitory action on acetylcholine packaging (Boroff, del Castillo, Evoy & Steinhardt, 1974). Whittaker (1974) has recently suggested that ATP is involved with vesiculin in the vesicular storage of acetylcholine. We suggest that an inhibition of oxidative phosphorylation by TPB may lead to a deficiency of ATP and thus interfere with acetylcholine storage in the synaptic vesicles.

The high m.e.p.p. frequency that appears after washing of TPB-treated tissues, and the evidence from studies on evoked transmitter release that the probability of release remains enhanced even after prolonged perfusion of TPB (Marshall & Parsons, 1975), suggests that small quantities of acetylcholine, producing effects indistinguishable from the noise, probably continue to be released, despite the apparent abolition of m.e.p p. activity. That the probability of transmitter release remains high, the quantum size falls towards zero, and perfusion of lanthanum or a higher concentration of TPB induces no further transmitter release, suggests that TPB depletes 'releasable' acetylcholine stores by a combination of rapid release plus inhibition of storage of transmitter.

The reversibility of the phenomenon indicates that the mechanism of action of TPB is different from that of black widow spider venom, that has been shown to deplete nerve terminals of acetylcholine and vesicles (Longenecker *et al*. 1970; Clark, Mauro, Longenecker & Hurlbut, 1970; Clark, Hurlbut & Mauro, 1972).

Recent preliminary morphological observations indicate that after 30 min exposure to TPB 0.05 mM a partial depletion (approximately 65%) of synaptic vesicles in individual nerve terminals occurs without a concomitant change in vesicle diameter (Marshall, Parsons & Paull, unpublished observation).

This work was supported by NIH Grant NS-07740 to RLP and was done during the tenure of a Research Fellowship of Muscular Dystrophy Associations of America, and a Wellcome Research Travel Grant to IGM. We also acknowledge the valuable assistance of Mr P. Spannbauer. Please send reprint requests to Dr R.L. Parsons in Vermont.

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(Received December 12, 1974
Revised March 11, 1975)

Br. J. Pharmac. (1975), 54, 339-349

AN INHIBITORY EFFECT OF ATROPINE ON RESPONSES OF THE VAS DEFERENS OF THE MOUSE TO FIELD STIMULATION

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1 Atropine is shown to impair responses of the vas deferens of the mouse to field stimulation by acting at a site proximal to the smooth muscle cells.

2 The inhibitory effect of atropine is prevented by desmethylimipramine and reversed by dexamphetamine, and appears similar to the adrenergic-neurone blockade of guanethidine.

3 Electronmicroscopical studies show the presence in vas of presumptive noradrenergic axons which have acetylcholinesterase reaction product associated with their axolemmae.

4 These results are discussed in relation to the controversial hypothesis of a 'cholinergic link' in noradrenergic transmission.

Introduction

Previous pharmacological studies of the vas deferens have been confined largely to organs obtained from guinea-pigs or rats and the innervation of these structures is considered to be adrenergic in nature (Huković, 1961; Birmingham & Wilson, 1963; Graham, Al Katib & Spriggs, 1968; Swedin, 1971; Furness, 1974). The existence of a physiologically important cholinergic component in the innervation was largely accounted for by the discovery of ganglionic relays in what had hitherto been considered as classical post-ganglionic sympathetic nerves (Sjöstrand, 1962; Graham *et al.*, 1968). Postganglionic neurotransmission is not sensitive to hemicholinium (Bentley & Sabine, 1963) and is resistant to blockade by atropine (Bentley & Sabine, 1963; Birmingham & Wilson, 1963; Graham *et al.*, 1968). There remain, however, phenomena which are inconsistent with the concept that the vas is a completely noradrenergically innervated tissue. Axons which show a positive reaction for acetylcholinesterase have been reported to be present in the vas of the guinea-pig (Robinson, 1969); the response of the vas to postganglionic nerve stimulation is potentiated by anticholinesterase drugs (Birmingham, 1966) and the sensitivity of the vas to noradrenaline (Ambache & Zar, 1970) and to α-adrenoceptor blockade is very low (Bentley & Smith, 1967).

In the present study, in which the vas deferens of the mouse was used, atropine exerted an inhibitory effect on responses to field stimulation. The possibility of a physiologically significant cholinergic component in the innervation of the vas of mouse has been investigated by means of pharmacological and morphological techniques.

Methods

Mature male albino mice, T.O. strain, weighing 25-35 g and over four weeks old were killed by cervical dislocation and both vasa deferentia removed. Single organs were suspended in Huković's (1961) solution of the following composition (mM): NaCl 113, KCl 4.6, $CaCl_2$ 2.5, KH_2PO_4 1.1, $MgSO_4$ 1.1, $NaHCO_3$ 25.0, glucose 11.5, bubbled with 5% CO_2 in O_2 at 32°C. The tissue was subjected to 300 mg applied tension and allowed to equilibrate for 30-40 minutes. Isometric responses were monitored on a Devices 2 channel pen recorder via a 2 oz dynamometer UFI strain gauge. Isotonic contractions were recorded on smoked paper with a frontal writing lever or on chart paper with an ink writing 'Follograph' (EOS Industrial Electronics Ltd. see Tonks & Williams, 1972). The magnification used in each isotonic method was 10 to 15 times.

Field stimulation (Birmingham & Wilson, 1963) of the vas, suspended between two parallel platinum wire electrodes immersed in a 50 ml bath, was by means of an SRI 6053 stimulator delivering rectangular pulses of 0.3 ms duration at frequencies of 30 Hz and of supramaximal voltage every 3.25 minutes. An oscilloscope was used to confirm the uniformity and reproducibility of the stimulation parameters under experimental conditions with the electrodes totally immersed in the bathing fluid.

Inhibitory drugs

The response to field stimulation was measured 20 min after exposure of the vas to the drug, at which time the inhibition was maximal.

Results are expressed in terms of percentage inhibition which was calculated as:

$$100 - \left(\frac{\text{size of response 20 min after exposure to drug}}{\text{size of (control) response before exposure to drug}} \times 100 \right).$$

The percentage inhibition caused by the inhibitory drug in the presence of dexamphetamine (Dex) or desmethylimipramine (DMI) was calculated as:

$$100 - \left(\frac{\text{size of response 20 min after exposure to guanethidine or atropine}}{\text{size of (control) response before exposure to Dex or DMI}} \times 100 \right)$$

Mean percentage inhibition with standard error was calculated for each dose of drug. The correlation coefficient, regression coefficient, and the corresponding regression line was determined for each experimental condition.

Responses to noradrenaline or acetylcholine

In experiments not involving field stimulation, tissues were suspended in 10 ml baths. Noradrenaline (NA), acetylcholine (ACh) or potassium was left in contact with the tissues for 0.5 min every 3 minutes. Bathing fluid was replaced at 1.5 min intervals. When testing the response to NA, ACh or potassium in the presence of an inhibitor a 15 min period elapsed before addition of agonist during which time the bathing fluid + inhibitor was changed every 1.5 minutes.

Analysis of covariance (Snedecor, 1956) was used to determine whether regression lines were parallel and coincident with one another.

Electron Microscopy

Mice were injected intravenously with vehicle (0.2 mg ascorbic acid/ml 0.9% w/v NaCl solution or 6-hydroxydopamine (6-OHDA) 0.2, 0.4 or 1 mmol/kg, killed 24 h later by cervical dislocation and both vasa deferentia removed.

Routine investigation One mm cubes of epididymal end of vas tissue were immersed in 1% osmium tetroxide, buffered to pH 7.5 with barbitone acetate for 1 h at 4°C, dehydrated and subsequently embedded in araldite. Fine sections were stained with lead citrate (Reynolds, 1963) and viewed with a Phillips 300 electron microscope.

Demonstration of acetylcholinesterase (AChE) Specimens were fixed in gluteraldehyde and incubated for 4 h at pH 5.5 with acetylthiocholine in the presence of 2×10^{-4} M ethopropazine (a pseudocholinesterase inhibitor) after the method of Lewis & Shute (1966). When physostigmine (2×10^{-4}M), a non-specific anticholinesterase was used in place of ethopropazine, no acetylcholinesterase reaction product was visible in any of the specimens examined.

Results

Field stimulation

Inhibition of responses to field stimulation was induced by each of the following drugs, guanethidine, phentolamine, pronethalol, lignocaine, atropine or hyoscine, when present in the bath in appropriate concentration (Figure 1). The inhibitory effect of lignocaine was readily reversed on washing out the bath, whereas the effects of the other drugs were not alleviated after repeatedly washing out the bath over a 30 min period. A relationship which did not differ from linearity ($P < 0.001$) was found between the log dose of each drug used and its inhibitory activity. There was no significant difference ($P > 0.05$) between regression coefficients and the regression lines may be considered to be parallel A significant difference in elevation indicates that the lines are not coincident ($P < 0.01$)

Responses to noradrenaline or acetylcholine

In the presence of guanethidine (2.0×10^{-6}M) or pronethalol (7.5×10^{-5}M) the response of the vas to NA (6×10^{-6}M) was unaffected or potentiated and that to ACh (2×10^{-4}M) was unmodified. Lignocaine (6.9×10^{-4}M) increased the response

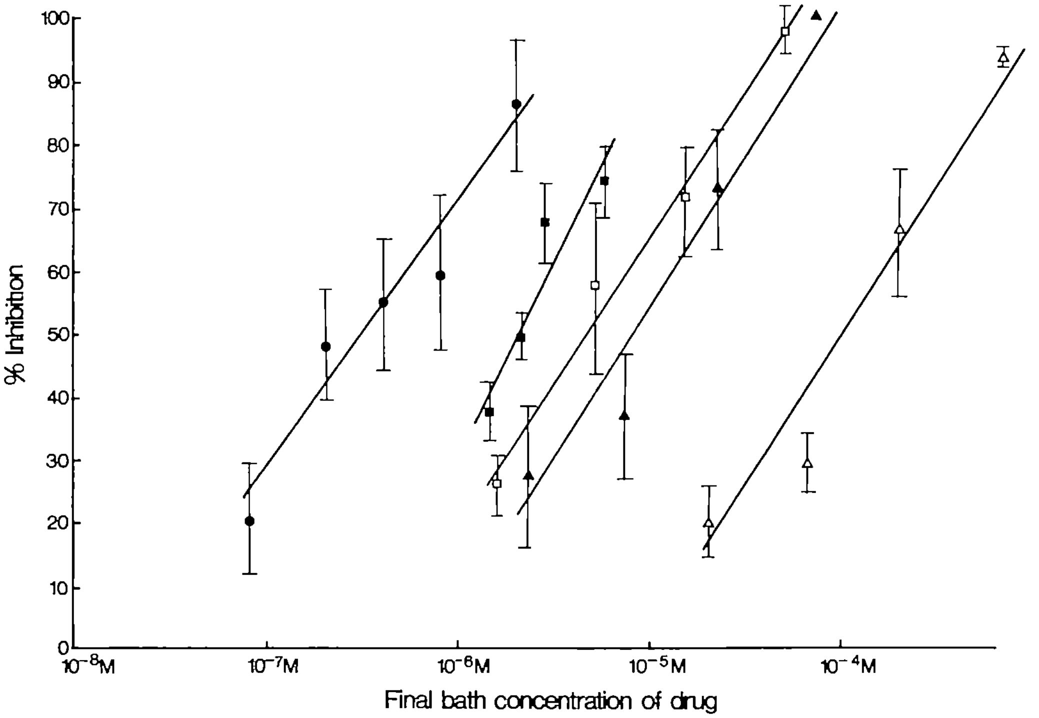

Figure 1 The percentage inhibition of the response of the vas deferens of the mouse to field stimulation in the presence of guanethidine (●), atropine (■), phentolamine (□), pronethalol (▲), or lignocaine (△). Each point is the mean ± s.e. of the values obtained from at least 5 vasa. The lines are drawn through co-ordinates calculated by the method of least squares analysis.

of the vas to NA or ACh but responses returned to control values after washing the lignocaine from the tissue bath. Phentolamine (5.3×10^{-5}M) abolished completely the response to NA which was not restored after repeated washings of the tissue with phentolamine-free Hukovič solution. The response to ACh was unaffected by the presence of phentolamine.

The response of the vas to ACh (1×10^{-4}M) was abolished in the presence of atropine (2.9×10^{-6}M) whereas that to potassium (2×10^{-2}M) and NA appeared unaffected The log dose-response curve for NA in the presence of atropine (2.9×10^{-6}M) was further investigated (Figure 2) in 12 vasa and found not to differ from linearity in the concentration range 3×10^{-7} to 1.2×10^{-4}M (correlation coefficient, $r = 0.96$, $d.f. = 130$, $P < 0.001$; regression coefficient, $b = 30.18$) and was compared with that for NA alone ($r = 0.93$, $d.f. = 117$, $P < 0.001$, $b = 32.56$). There was no significant difference ($P > 0.05$) in the slopes or elevations of these two lines.

The antagonism of drug-induced blockade of nerve transmission

DMI (9.4×10^{-8}M) or dexamphetamine (2.7×10^{-6}M) added to the bath prior to guanethidine inhibited the development of guanethidine-induced blockade (Figure 3). Atropine-induced blockade similarly was prevented by prior administration of DMI or dexamphetamine (Figure 4).

For each preparation the percentage inhibition caused by the inhibitory drug in the presence of DMI or dexamphetamine was calculated. A relationship which did not differ from linearity ($P < 0.001$) was found between this percentage inhibition and concentration of guanethidine or atropine in the bath (Figures 3 and 4). An analysis of covariance carried out on the regression lines showed no significant difference ($P > 0.05$) between the slopes of the six regression lines, and significant ($P < 0.01$) shifts of the log dose-response curve for guanethidine to the right in the

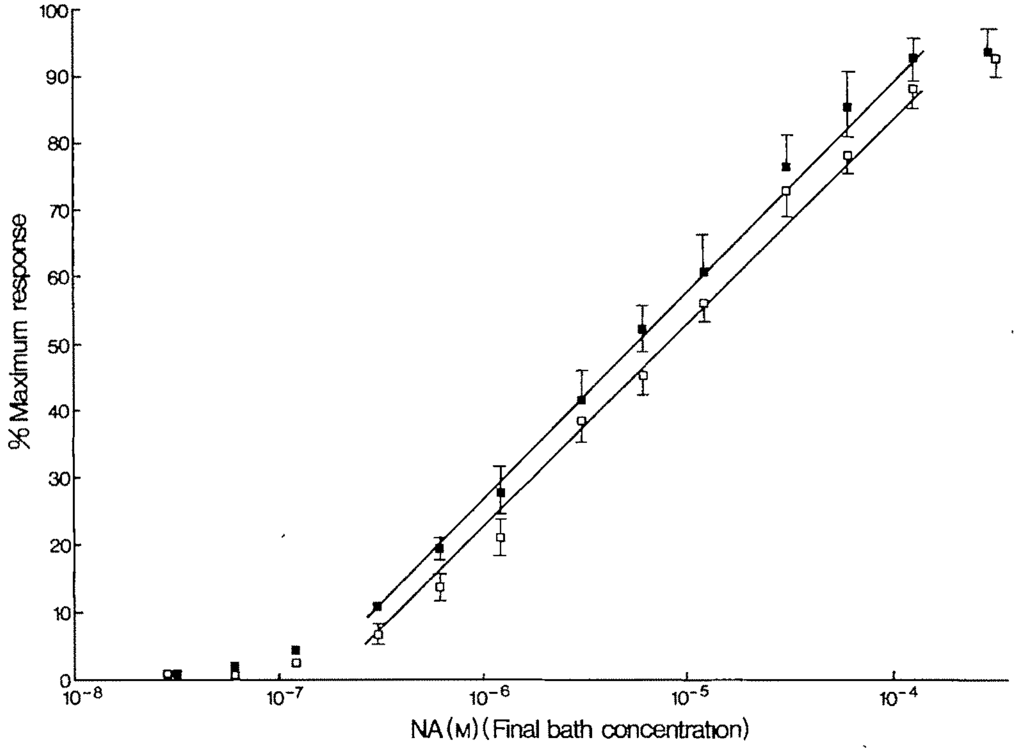

Figure 2 The log dose-response curve for noradrenaline (NA) alone (■), and NA in the presence of atropine (□, 2.9×10^{-6}M) in the vas deferens of the mouse. Each point is the mean ± s.e. from 12 vasa. The lines are drawn through co-ordinates calculated by the method of least squares analysis in the dose range 3×10^{-7} M to 1.2×10^{-4} M for NA.

Table 1 Quantitation of the inhibitory effect of guanethidine, atropine, hyoscine or lignocaine on the response of the vas deferens of the mouse to field stimulation, and the effect of dexamphetamine thereon.

Drug	*Final bath conc. M*	*Inhibited response as % control response*	*Response after dexamphetamine (1.1×10^{-5} M) as % control response*
Guanethidine	8.1×10^{-8}	79.4 ± 8.4 (6)	102.0 ± 5.5 (6)*
	2.0×10^{-7}	51.9 ± 8.8 (6)	102.5 ± 14.9 (6)**
	4.0×10^{-7}	45.4 ± 10.2 (6)	90.8 ± 6.7 (6)**
	8.1×10^{-7}	40.2 ± 12.2 (6)	93.5 ± 8.9 (6)**
	2.0×10^{-6}	13.5 ± 10.2 (5)	90.8 ± 13.2 (5)**
Atropine	1.4×10^{-6}	62.2 ± 4.0 (7)	105.5 ± 10.3 (7)**
	2.2×10^{-6}	50.4 ± 3.6 (6)	79.0 ± 8.0 (6)**
	2.9×10^{-6}	32.2 ± 6.4 (6)	87.4 ± 9.2 (6)**
	5.8×10^{-6}	25.6 ± 5.5 (6)	77.7 ± 3.1 (6)**
Hyoscine	1.4×10^{-6}	63.1 ± 5.4 (3)	91.2 ± 2.9 (2)**
Lignocaine	2.1×10^{-5}	80.0 ± 5.4 (6)	93.6 ± 27.48 (3)
	6.9×10^{-5}	70.9 ± 4.9 (6)	82.3 ± 9.78 (4)
	2.1×10^{-4}	43.6 ± 6.7 (6)	59.1 ± 1.52 (2)

Significant differences between columns 2 and 3 were calculated using Student's *t* test (Saunders & Fleming, 1957) $^*P < 0.05$; $^{**}P < 0.01$.

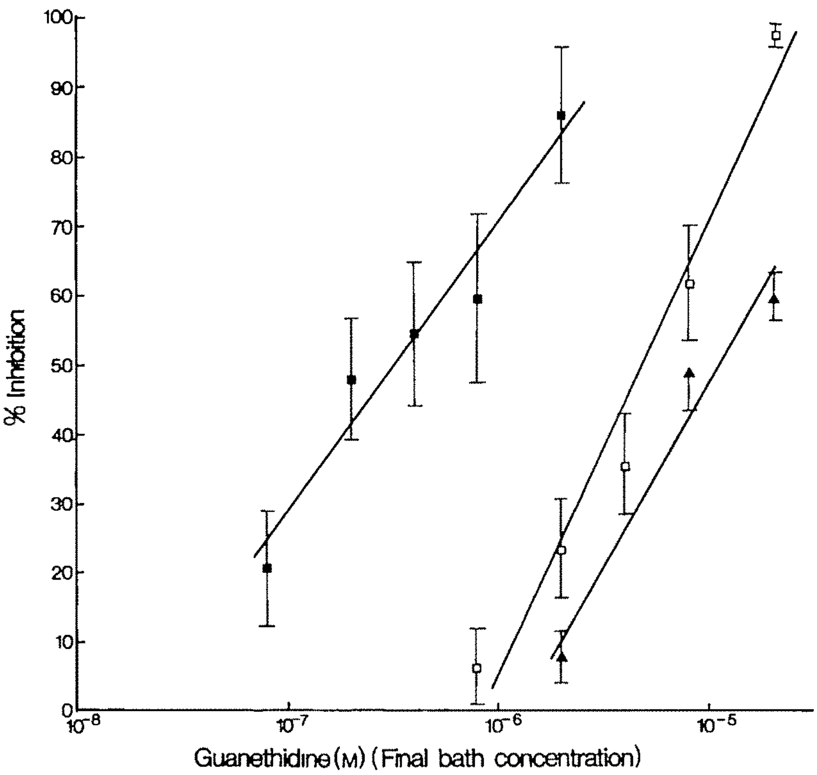

Figure 3 The inhibition by dexamphetamine (2.7×10^{-6}M) or desmethylimipramine (DMI) (9.4×10^{-8}M) of the guanethidine-induced blockade of responses of mouse vas to field stimulation. Responses are plotted as the percentage inhibition of the maximum response to field stimulation. Each point is the mean ± s.e. from not less than 4 vasa. The lines are drawn through co-ordinates calculated by the method of least squares analysis. (■), Guanethidine alone; (□) DMI + guanethidine; (▲) dexamphetamine + guanethidine.

presence of DMI or dexamphetamine. The shifts in the log dose-response curve for atropine in the presence of DMI or dexamphetamine are also significant ($P < 0.01$).

The reversal of drug-induced blockade of nerve transmission

The blockade induced by guanethidine, atropine or hyoscine was reversed significantly ($P < 0.05$) by the addition of dexamphetamine (1.1×10^{-5}M) to the bath. Dexamphetamine did not reverse the inhibitory effect of lignocaine, phentolamine or pronethalol. In Table 1 responses, measured 20 min after guanethidine (or atropine) and responses measured 20 min after the additional administration of dexamphetamine, have been expressed as percentage of control (pre-guanethidine or pre-atropine) responses.

Electron microscopy

Axons containing small dense-core vesicles (25-60 nm) in tissue fixed with glutaraldehyde and post-osmicated are characteristic of noradrenergic axons and accounted for 85% of the total axon population. In tissues fixed solely with OsO_4, only 41% of axons possessed small dense-core vesicles (Table 2). However, previous workers have reported that OsO_4 is not a good fixative for dense-core vesicles (Richardson, 1962; Tranzer, Thoenen, Snipes & Richards, 1969). In tissue pretreated with 6-OHDA presumptive noradrenergic axons show small dense-core vesicles even with OsO_4 fixation or (with larger doses) clumping of axoplasmic contents to form dense osmiophillic masses (Table 2).

In tissue that had been incubated with acetylthiocholine, fixed in gluteraldehyde and

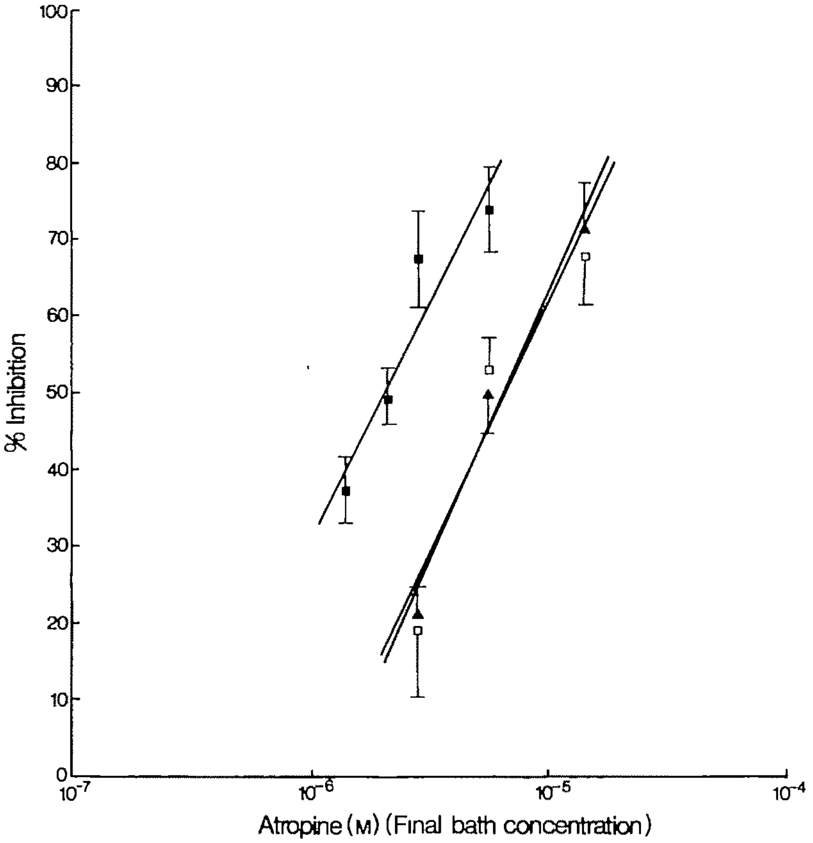

Figure 4 The inhibition by dexamphetamine (2 7 x 10^{-6}M) or desmethylimipramine (9.4 x 10^{-8}M) of the atropine-induced blockade of responses of mouse vas to field stimulation. Responses are plotted as a percentage inhibition of the maximum response to field stimulation. Each point is the mean ± s e. from not less than 5 vasa. The lines are drawn through co-ordinates calculated by the method of least squares analysis. (■) Atropine alone, (□) DMI + atropine; (▲) dexamphetamine + atropine.

Table 2 Analysis of electronmicrographs. the percentage of total axons having the axonal characteristic described in column 1.

Pretreatment of mice	None	None	6-OHDA 0.2 mmol/kg 24 h	6-OHDA 0.4 mmol/kg 24 h	6-OHDA 1 mmol/kg 24 h
Fixation of vas	Glutaraldehyde post OsO_4	OsO_4	OsO_4	OsO_4	OsO_4
Axonal characteristic					
1. Small dense-core vesicle	85 0	41.4	77.2	82.3	8.4
2. Small electron-translucent vesicle	9.3	50.0	10.0	6.1	7.2
3. Signs of degeneration	—	—	8.4	3.6	78.4
4 None of above characteristics	5.7	8.6	4 4	8.0	—

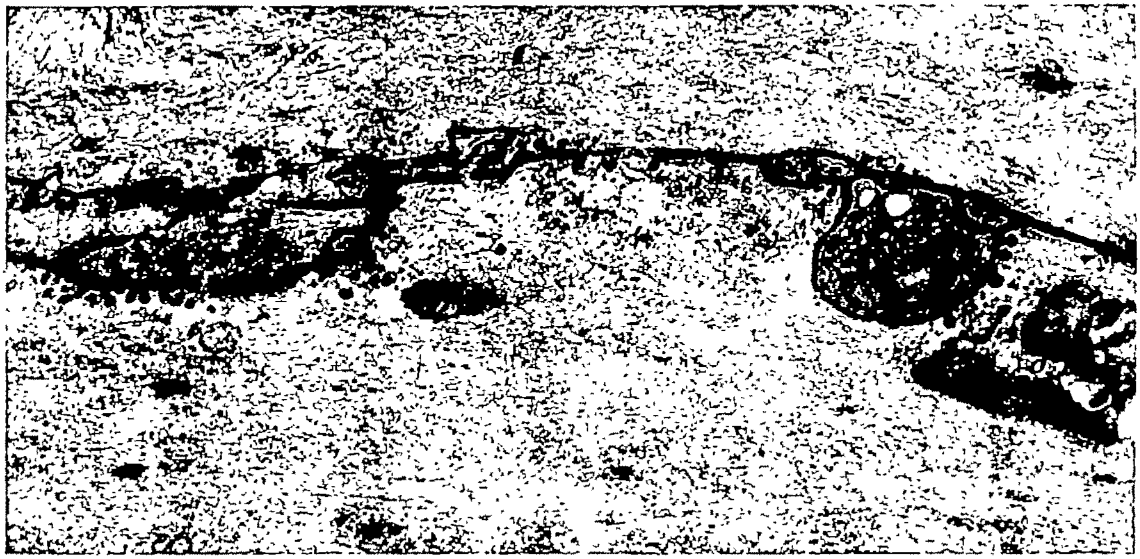

Figure 5 Electronmicrograph of glutaraldehyde-fixed vas incubated with acetylthiocholine. Two axons having an electron dense acetylcholinesterase reaction product associated with their axolemmae possess small vesicles (ca. 50 nm diameter) which contain electron-dense material. Such vesicles in glutaraldehyde-fixed tissue are indicative of noradrenergic nerves.

Table 3 The distribution of acetylcholinesterase (AChE) reaction product around axons in control and chemically sympathectomized vasa

	No. axons, with characteristics shown in column 1, as percentage of total axons	
Axon characteristics	*Control vasa**	*Chemically sympathectomized vasa*†*
1. Intact containing small dense-core vesicles with AChE reaction product	29.5	0
2. Degenerating with AChE reaction product	0	16.7
3. Intact with electron translucent vesicles and AChE reaction product	7.5	7.8
4. Intact with electron-translucent vesicles and *no* AChE reaction product	1.8	5.0
No. of axons studied	161	258

* Fixation with glutaraldehyde and post-osmicated
† Mice given 6-OHDA 1 mmol/kg intravenously 24 h previously.

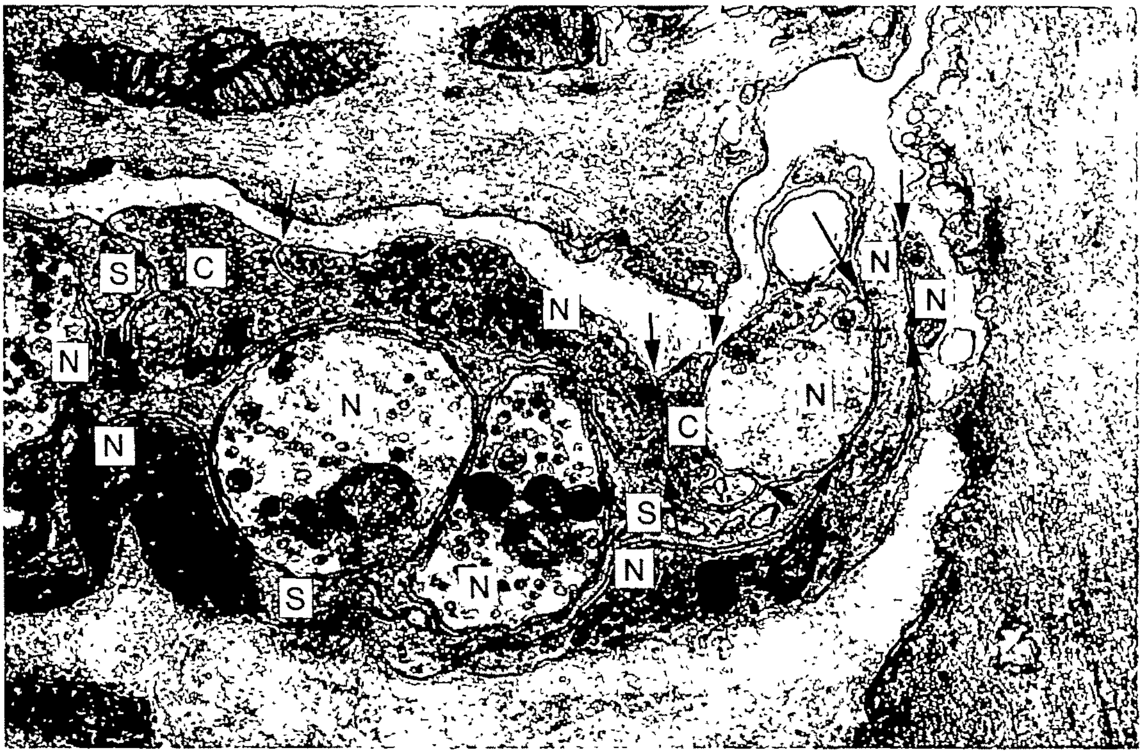

Figure 6 Electron micrograph of axon bundle in vas taken from mouse treated 1 h previously with 6-hydroxydopamine 0.2 mmol/kg. Presumptive noradrenergic axons (N) show conspicuous dense core vesicles, whereas presumptive cholinergic axons (C) contain electron-translucent vesicles Possible synaptic relationships between axons are indicated by arrows, the interval between axolemmae being ca. 20 nm and devoid of Schwann cell processes (S).

post-osmicated, acetylcholinesterase (AChE) reaction product was observed on the axolemma of most nerves containing small electron-translucent vesicles in both control or chemically sympathectomized vasa (Table 3, line 3). Such AChE positive axons constituted 7-8% of the total number of axons investigated, and are designated as presumptive cholinergic axons.

A χ^2 test (Seigal, 1956) showed that there was no difference in the population of AChE positive axons containing electron-translucent vesicles ($P > 0.05$) as between normal and sympathectomized vasa.

Because of the small sample number of AChE negative axons containing electron translucent vesicles in control vasa (Table 3, line 4) it was not possible to carry out χ^2 tests on this sample.

In addition to axons containing electron-translucent vesicles, AChE reaction product was observed on the axolemmae of axons containing small dense-core vesicles (Figure 5), 29.5% of the total number of axons studied in control vasa were AChE positive and contained small dense-core vesicles. In sympathectomized vasa 16.7% of the total showed signs of degeneration and were also AChE positive (Table 3). A χ^2 test showed that normal and sympathectomized vasa differed with respect to the number of presumptive noradrenergic axons which were also AChE positive ($P < 0.05$). Nerve bundles (containing 5.6 ± 0.24 axons/bundle) in the extracellular spaces within muscle cell bundles contained some axons which were densely packed with vesicles and mitochondria and others which contained only neurotubules. The Schwann cell sheath was often incomplete leaving some axolemmae naked in parts. Axons in bundles were frequently in close apposition (20 nm) to other axons within that bundle, a situation in which synaptic transmission could occur. In a survey of 475 axons in bundles, 84 presumptive noradrenergic axons and 8 presumptive cholinergic axons were juxtaposed to neighbouring axons and although no specializations of axolemmae were observed the relationships appeared potentially synaptic (Figure 6).

Discussion

The effective motor innervation of the vas deferens of the mouse appears to be exclusively noradrenergic in nature (Furness, 1974; Jones & Spriggs, 1975). The ability of guanethidine to block completely the response of the vas to field stimulation contributes to this hypothesis. An apparent inconsistency arises, however, with the present finding that atropine impairs responses to field stimulation. Although the concentrations of atropine used in the present study may appear large, they are comparable with the concentrations of ACh (2.8×10^{-6}M to 4.4×10^{-4} M) necessary to elicit contractions of the mouse vas (Jones & Spriggs, 1971) and do not impair responses to potassium (Jones, 1974) or to noradrenaline (Figure 2). Two possibilities arise:

1 *ACh is an important motor transmitter acting at muscarinic receptors on smooth muscle of the vas.* In support is the observation that presumptive cholinergic axons (AChE positive axons with electron-translucent vesicles) are present in the vas. However, such axons constitute less than 10% of the total axon population and although they survive chemical sympathectomy with 6-OHDA, transmural stimulation no longer elicits any increase in tension of the vas (Jones & Spriggs, 1975).
2. *Atropine impairs sympathetic transmission and/or smooth muscle contractility in the vas of the mouse.* A post-synaptic action is unlikely as the response of the vas to potassium remained unimpaired by the presence of atropine in concentrations which inhibited contractions induced by field stimulation; responses to exogenous NA were similarly unaffected.

Muscarinic receptors on adrenergic neurones in the heart have been reported by Lindmar, Löffelholz & Muscholl (1968). These receptors, when stimulated by ACh antagonize the release of NA. The activity of atropine described in this paper cannot be explained by the presence on noradrenergic neurones of this type of muscarinic receptor.

Percentage inhibition of responses to field stimulation/log dose plots for atropine and guanethidine are parallel, not only to each other, but also to similar plots for the local anaesthetic lignocaine, the α-adrenoceptor blocker phentolamine and the β-adrenoceptor blocker pronethalol. The inhibitory effect of atropine was persistent, as was that of guanethidine, in contrast to that of lignocaine which was readily reversed on washing out the bath.

Dexamphetamine is effective in reversing the adrenergic neurone blockade of guanethidine and similar drugs in many tissues (Day & Rand, 1963; Spriggs, 1966) including the vas of the mouse. In the present experiments dexamphetamine also reversed the inhibitory effect of atropine, but not that of lignocaine, phentolamine or pronethalol. Thus the inhibitory action of atropine is more like that of guanethidine than that of any of the other drugs used. This similarity was upheld in further experiments in which pretreatment of the vas with the uptake$_1$ inhibitor DMI significantly reduced the inhibitory effect of atropine as it did that of guanethidine (Figures 3 and 4). The possibility arises that atropine may be interfering with a 'cholinergic link' in noradrenergic transmission (Burn & Rand, 1959). Most of the evidence used in support of this controversial hypothesis would favour the involvement of 'nicotinic-like' rather than 'muscarinic-like' receptors (Burn & Rand, 1959, 1965; Cabrera, Torrance & Viveros, 1966, Rand & Wilson, 1967). On the other hand, Kadzielawa & Gumulka (1967a, b) and more recently Lederer, Rand & Wilson (1970) have claimed similarities between the adrenergic-neurone blocking activity and the anti-muscarinic potency of some compounds which they have investigated. However, we are not satisfied that their experimental design or the statistical appraisal of their results justifies such conclusions.

The finding that almost 40% of presumptive noradrenergic axons (i.e. axons containing dense-core vesicles in gluteraldehyde-fixed tissue or after 6-OHDA treatment) in the vas of the mouse showed a positive reaction for AChE is compatible with the 'cholinergic-link' hypothesis. However, acceptance of this hypothesis leaves unresolved the ability of atropine (5.8×10^{-6}M) to cause 75% inhibition of responses to field stimulation when only 40% of the presumptive noradrenergic axons show a positive reaction for AChE.

It has been argued that the presence of AChE is a prerequisite for a functional cholinergic mechanism and in earlier work on noradrenergic neurones (Esterhuizen, Graham, Lever & Spriggs, 1968; Graham, Lever & Spriggs, 1968) the absence of AChE was considered to militate against the presence of a functional cholinergic mechanism. The present findings of AChE in relation to presumptive noradrenergic axons has prompted us to undertake firstly, a more rigorous assessment of the specificity of this AChE and secondly, autoradiographic evidence that these axons do take up [^{3}H]-noradrenaline. However, we consider that there is now sufficient evidence for the 'classical noradrenergic neurone' to undergo careful scrutiny in each different tissue, and that one should be cautious in making or interpreting generalized statements concerning noradrenergic nerves; there are now reports in the literature of tissues in which (1) *no* noradrenergic axons show AChE 'staining' (pancreatic arterioles, Graham *et al.*, 1968; and nictitating muscles, Esterhuizen *et al.*, 1968, of cat) (2) *some* noradrenergic axons show AChE 'staining' (hypogastric and splenic nerves of cat, Lever, Spriggs, Graham & Ivens, 1970; vas of rat, Mottram, Ivens, Lever & Presley, 1973, vas of mouse, this paper) (3) *all* noradrenergic axons show AChE 'staining' (pineal of rat, Eranko, Rechardt, Eranko & Cunningham, 1970).

M.E.L.J. undertook this work during tenure of a Thomas and Elizabeth Williams Scholarship and gratefully thanks the Carmarthenshire C.C. Education Committee who made the award.

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(Received February 3, 1975.
Revised April 11, 1975.)

Br. J. Pharmac. (1975), 54, 351-358

INNERVATION BOTH OF PERI-ORBITAL STRUCTURES AND OF THE HEART BY THE CERVICAL SYMPATHETIC NERVES IN MOUSE, RAT, GUINEA-PIG, RABBIT AND CAT

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1 In anaesthetized rats electrical stimulation of the intact cervical sympathetic nerve produced frequency-dependent lower eyelid contractions and tachycardia.

2 The tachycardia was caused by excitation of efferent fibres since it was equally evident in the pithed rat preparation, and the right nerve was more effective than the left. By contrast, no differences were seen between the responses to right and left vagal stimulation in either rats or rabbits.

3 Guanethidine inhibited both cardiac and eyelid responses, propranolol only the former and phentolamine only the latter, thereby revealing the adrenergic nature of the nerves. Hexamethonium caused partial inhibition and the block was intensified by atropine.

4 The inferior eyelid of mice, guinea-pigs and rabbits as well as the nictitating membrane of rabbits and cats were contracted by cervical sympathetic nerve stimulation. In these species too, tachycardia occurred; this was more pronounced with the right than the left sympathetic nerve. The order of cardiac responsiveness was mouse > rat > guinea-pig > rabbit > cat.

5 In guinea-pigs histamine-induced bronchoconstriction was reduced by cervical sympathetic nerve stimulation.

6 That discrete cardiac pathways exist in the cervical sympathetic nerves is suggested by the reproducibility of the effects within any one species. The accessibility of the nerves greatly simplifies the examination of drugs *in vivo* on two different structures innervated by the sympathetic nervous system.

Introduction

It is generally accepted that the cervical sympathetic nerves convey fibres from the thoracic sympathetic outflow to the superior cervical ganglion from which post-ganglionic neurones predominantly supply structures in the head and neck. Equally the major pre- and post-ganglionic components of the cardiac sympathetic nerves are thought to lie in the chest.

Large (1974) described experiments which showed that stimulation of the cervical sympathetic nerves in rats produced not only retraction of the appropriate eyelid but also marked tachycardia. The results presented here extend the observations in rats and also demonstrate that cardioaccelerator fibres are similarly excited in mice, guinea-pigs, rabbits and cats.

Methods

Experiments in anaesthetized rats

Male Wistar rats, weighing 250-350 g, were anaesthetized with a mixture of chloralose (100-120 mg/kg) and sodium pentobarbitone (30 mg/kg) injected through a nylon cannula terminating in a 17 G needle shaft dwelling in a tail vein.

In some experiments the left iliac, in others the left common carotid artery just below the bifurcation into external and internal branches, was cannulated for blood pressure measurement, the pressure wave being used to trigger a ratemeter. A thread was tied to the right inferior eyelid (or the left in 3 experiments) and connected to a Dynamometer UF1 transducer to record isometric tension changes with an initial tension of 0.4 to 0.5 g. A tracheal cannula was inserted and artificial respiration was maintained with a Miniature Starling pump, rate 72/min and stroke volume 10 ml/kg body weight.

Each vagus in the neck was doubly ligated and sectioned. The cervical sympathetic nerves were left intact and placed on shielded bipolar platinum electrodes immersed in liquid paraffin to avoid current-spread. Each nerve was usually stimulated at a position about 8 mm caudal to the superior cervical ganglion, although a position 15 to 20 mm was chosen for the left nerve in experiments where a cannula had been placed in the left carotid artery in case the blood vessels supplying the nerve had been damaged. Occasionally the electrode position was altered during an experiment to discover if all the fibres stimulated ran the visible length of the cervical sympathetic nerve. The cathode was caudal to the anode in all but the first 3 experiments when reversal of the polarity was shown not to affect the sizes of the responses. The nerve was stimulated with rectangular pulses, 0.5ms duration, supramaximal voltage (usually 8-10 V) and various frequencies. The frequency-response curves were obtained by initially stimulating at a low frequency, 0.1 or 0.2 Hz, and, after a plateau appeared in the responses, by progressively increasing the frequency to 0.5, 1, 2, 5, 10 and 20 Hz. Similar sequences of stimulation were applied to the cardiac ends of the severed vagi in 5 experiments. All recordings were made on a Devices M-19 recorder.

In a further 6 experiments, following injection of the anaesthetic and after applying artificial respiration, rats were pithed by introducing a steel rod through the left orbit and passing it down the spinal cord. The rod was left *in situ* during the experiment and destruction of the spinal cord was verified by subsequent dissection. In this group of animals the conditions were identical to those previously described with the exception that all blood pressure measurements were made from the carotid artery and the initial eyelid tension was 0.7 to 0.8 g.

The experimental procedures employed in the other species were similar to those in rats with the following exceptions.

Male guinea-pigs, body weight 480-520 g, were anaesthetized with an intraperitoneal injection of chloralose (100 mg/kg) and sodium pentobarbitone (30 mg/kg). The blood pressure was always taken from the left carotid artery, tension changes were measured in the right inferior eyelid only at an initial tension of 1 g and intravenous injections were given through a cannulated left jugular vein. Changes in inflation pressure during artificial respiration were recorded through a pressure transducer connected to the tracheal cannula.

Male mice, Tuck No. 1 strain, weighing 35 to 40 g were used. The most suitable anaesthetic mixture for a stable preparation was found to be chloralose (100-120 mg/kg) and sodium hexobarbitone (100 mg/kg) which was given intravenously through nylon tubing attached to a needle shaft lodged in a tail vein and subsequently used for drug administration. Artificial respiration was given at a rate of 100 strokes/min and the pump set at approximately 1 ml stroke volume, a final adjustment to the latter being made such that the resting blood pressure did not alter by more than 5 mmHg from the level recorded during spontaneous respiration. Following bilateral vagotomy, the right sympathetic nerve only was stimulated. Heart rates were measured by increasing the chart speed during stimulation periods and counting the individual pulses at a time 50 s after the frequencies were changed. Right inferior eyelid contractions were measured from a resting tension of 150-200 mg. Blood pressure was recorded from the left common carotid artery.

Male cats weighing 1.9 to 2.5 kg received chloralose (100 mg/kg) and sodium pentobarbitone (6 mg/kg) intraperitoneally. They were artificially respired at 20 strokes/min with stroke volume 12.5 ml/kg, blood pressure was taken from the left femoral artery and right nictitating membrane contractions were recorded isometrically at a resting tension of 1.5 g. Injections were made through a cannula in the left femoral vein.

Male albino rabbits, weighing 2.5 to 3.0 kg, were anaesthetized with 100 mg/kg chloralose and 30 mg/kg pentobarbitone sodium given via an ear vein; an indwelling needle served for injections given during the experiment. Blood pressure was recorded from the left carotid artery, cannulated near the bifurcation into internal and external carotids. The tension changes were recorded from either the right nictating membrane (initial tension 0.5 g) or the right inferior eyelid (1.5 g). Artificial respiration was given at 40 strokes/min, stroke volume 10 ml/kg body weight.

The following drugs were used: atropine sulphate, guanethidine monosulphate (Ismelin, Ciba Laboratories Ltd), hexamethonium bromide (Koch-Light Laboratories Ltd), histamine acid phosphate (BDH Chemicals Ltd), phentolamine mesylate (Rogitine, Ciba Laboratories Ltd), and (±)-propranolol hydrochloride (Sigma Chemical Company). All doses of drugs are expressed as the salt.

Results

Effects of vagal or sympathetic nerve stimulation in anaesthetized rats

There was no significant difference in the bradycardia evoked by stimulating either the left or right peripheral vagus. Frequency-response curves were virtually superimposable; cardiac arrest occurred in all 5 rats when 50 Hz was applied to the left vagus and in 4 out of 5 rats when the right nerve was stimulated.

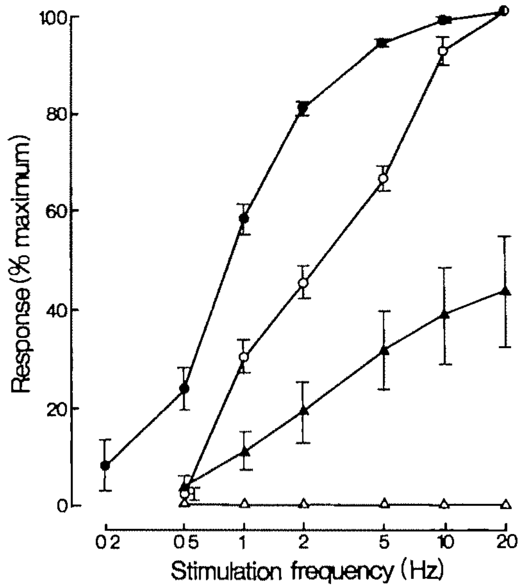

Figure 1 Effects of electrical stimulation of the intact right or left cervical sympathetic nerves on heart rate and on contractions of the right inferior eyelid in anaesthetized rats. Responses are expressed as percentages of the maximal effects caused by stimulation at 20 Hz and are means of determinations in 6 rats; vertical lines show s.e. mean. The nerves were stimulated with rectangular pulses at supramaximal voltage (8-10 V), 0.5 ms pulse width and at progressively increasing frequencies which were changed as soon as a plateau occurred in the responses. Tachycardia on stimulating the right nerve (●) or the left nerve (▲); eyelid contraction with the right nerve (○) or the left nerve (△).

Stimulation of the cervical sympathetic nerve causes retraction of the inferior eyelid (Gertner, 1956). In the present experiments the tension changes in the right eyelid were similar in magnitude when either the intact ipsilateral nerve or its cephalic end after sectioning was stimulated. The minimum effective frequency was usually 1 Hz, maximum tension changes occurred at 10 or 20 Hz and the eyelid was seldom able to maintain its contraction at the latter frequency (Table 1), complete recovery occurred within 60 seconds. The left eyelid tension only was measured in 3 further rats and frequency response curves were found to be virtually identical to those from the right side.

Tachycardia also occurred when the intact right cervical sympathetic nerve was stimulated, an effect which was abolished if the nerve was sectioned caudal to the electrode but which remained unaltered if the nerve was severed cephalad to it. The heart was more sensitive than the eyelid to low frequency stimulation and the tachycardia was nearly maximal with 5 Hz (Table 2). The heart rate often did not recover until 3 to 8 min after ceasing stimulation.

When the left sympathetic nerve was stimulated, frequencies below 1 Hz were ineffective and 20 Hz was needed for maximum tachycardia. The greatest tachycardia measured was invariably smaller than the corresponding response from the right nerve (usually less than 50%) and is illustrated in Figure 1.

The blood pressure records provided a further contrast between the two nerves. When the right one was stimulated mean pressure frequently rose, especially when the tachycardia exceeded 100 beats/min, but pulse pressure rarely changed. By contrast stimulation of the left nerve invariably raised the pulse pressure, occasionally in the absence of tachycardia. In most experiments the pulse pressure was doubled in size when a frequency of 20 Hz was used, but no precise measurements were possible since a slow recording chart speed was employed. Typical responses are illustrated in a later section.

Effects of sympathetic nerve stimulation in pithed rats

When calculated as a percentage of the maximum response to 20 Hz the responses of the right eyelid to nerve stimulation were indistinguishable from those recorded in anaesthetized rats (illustrated in Figure 1). The absolute tension changes were, however, about 30% higher than the previous measurements. These differences may be attributable to the use of a higher initial eyelid tension in the pithed rat preparation.

The cardiovascular changes were similar in most respects to those recorded in the anaesthetized rats except that an increased sensitivity of the heart to low frequency stimulation of the right nerve became apparent (Figure 2). Tachycardia now followed the application of a single shock and 0.1 Hz increased the heart rate by 50 beats/min in 2 experiments. The detailed results are included in Tables 1 and 2.

Effects of sympathetic nerve stimulation in guinea-pigs

The inferior eyelid of the guinea-pig receives an innervation from the cervical sympathetic nerve as does the rat. Contractions of the right eyelid calculated as percentage of the maximum were similar to those in rats when the frequency-response curves were determined Absolute

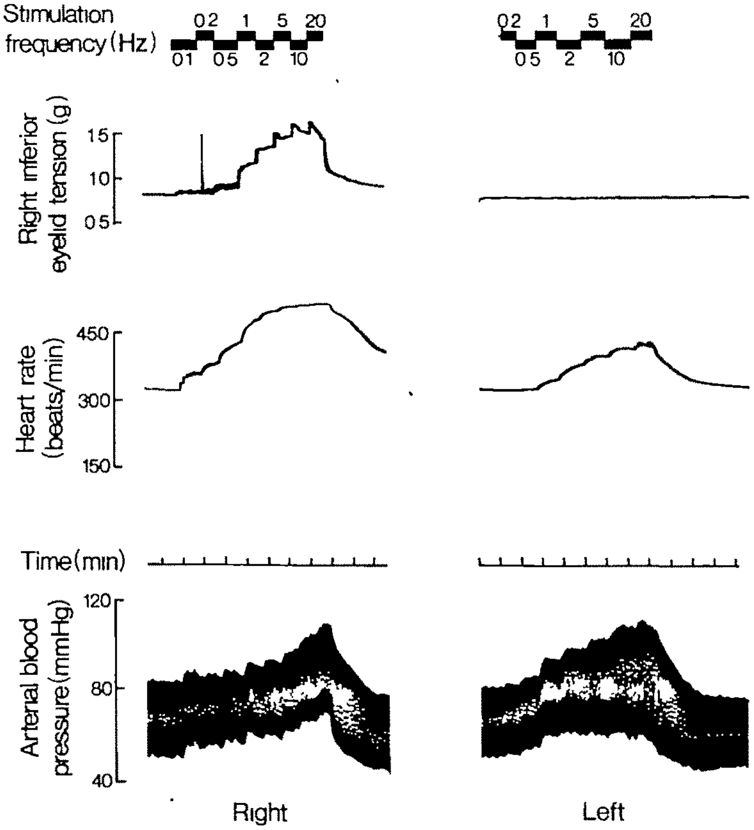

Figure 2 Effects of electrical stimulation of the intact cervical sympathetic nerves in a pithed rat preparation, using progressively increasing frequencies, supramaximal voltage (10 V) and 0.5 ms pulse width. Note particularly that the eyelid responds only to ipsilateral nerve stimulation, the tachycardia is greater when the nerve on the right is stimulated, and the pulse pressure increases more when the left nerve is stimulated.

Table 1 Isometric tension changes in the right inferior eyelid of anaesthetized mice, rats, guinea-pigs and rabbits, or nictitating membrane of cats and rabbits when the intact right cervical sympathetic nerves were electrically stimulated.

Species	*Mouse*	*Rat*	*Pithed rat*	*Guinea-pig*	*Cat*	*Rabbit*	
No. in group	5	6	6	7	3	2	2
						Eyelid	*Nictitating Membrane*
Initial tension	0.1 – 0.15	0.4 – 0.5	0.7 – 0.8	1.0 – 1.2	1.5	1.0	0.5
Frequency (Hz)							
0.5	0	0	0.08 ± 0.02	0.09 ± 0.02	1.3 ± 0.4	0.1; 0.35	0.1, 0.08
1	0.02 ± 0.01	0.16 ± 0.02	0.23 ± 0.04	0.27 ± 0.03	3.0 ± 0.9	0.48; 0.75	0.16, 0.18
2	0.06 ± 0.03	0.30 ± 0.02	0.38 ± 0.05	0.52 ± 0.03	4.7 ± 1.1	0.72, 1.25	0.20; 0.25
5	0.16 ± 0.02	0.42 ± 0.03	0.56 ± 0.06	0.86 ± 0.05	6.2 ± 1.3	1.08; 1.80	0.35, 0.28
10	0.28 ± 0.04	0.50 ± 0.04	0.67 ± 0.06	1.12 ± 0.06	7.8 ± 1.5	1.40, 2.30	0.40; 0.32
20	0.33 ± 0.02	0.54 ± 0.04	0.72 ± 0.05	1.20 ± 0.08	10.3 ± 1.4	1.20; 2.20	0.30, 0.24

The values are mean tension changes (g) ± s.e. mean except for the results in the rabbit which are from separate experiments.

Table 2 Comparison of tachycardias produced in several species by electrical stimulation of the intact right or left cervical sympathetic nerves.

Nerve	Right						Left				
Species	*Mouse*	*Rat*	*Pithed rat*	*Guinea-pig*	*Rabbit*	*Cat*	*Rat*	*Pithed rat*	*Guinea-pig*	*Rabbit*	*Cat*
No in group	5	6	6	7	5	3	6	6	7	5	3
Initial heart rate	520 ± 16	299 ± 8	324 ± 4	216 ± 6	300 ± 10	183 ± 7					
Frequency (Hz)											
0.1			23 ± 7								
0.2		13 ± 6	48 ± 10								
0.5	22 ± 2	43 ± 9	82 ± 12	4 ± 1	0	0	5 ± 3	6 ± 5	0		
1	80 ± 7	105 ± 9	125 ± 9	26 ± 3	3 ± 2	2 ± 2	19 ± 7	15 ± 9	3 ± 2	0	0
2	130 ± 13	145 ± 8	143 ± 9	47 ± 5	13 ± 3	5 ± 3	33 ± 12	28 ± 15	7 ± 2	4 ± 2	2 ± 2
5	198 ± 9	168 ± 8	152 ± 10	72 ± 4	32 ± 4	13 ± 9	55 ± 15	39 ± 15	21 ± 5	6 ± 2	3 ± 3
10	223 ± 13	177 ± 7	153 ± 10	91 ± 4	44 ± 5	20 ± 9	68 ± 19	54 ± 19	33 ± 6	14 ± 10	8 ± 4
20	225 ± 14	179 ± 7	153 ± 10	102 ± 4	43 ± 4	22 ± 6	76 ± 20	56 ± 20	39 ± 6	18 ± 8	12 ± 6

Values are mean tachycardia (beats/min) ± s.e. mean

tensions were different since an initial tension of 1 g was employed and stimulation at 20 Hz produced a mean maximum increase of 1.20 g (Table 1).

When the intact sympathetic nerves were stimulated tachycardia occurred, more so with the right than the left. The absolute change in heart rate was smaller in this species; for instance, when the right nerve was stimulated at 20 Hz a tachycardia of 115 beats/min was never exceeded (Table 2).

In two guinea-pigs resistance of the lungs to inflation was also measured and intravenous histamine was periodically given to increase inflation pressure. Stimulation of the right sympathetic nerve, beginning 60 s prior to histamine injection, caused a frequency-dependent inhibition of the induced bronchoconstriction (about 80% reduction at 20 Hz) which was less pronounced if stimulation began 5 s before the injection. This 'histamine antagonism' may have involved sympathetic bronchodilator fibres in the cervical nerve.

Effects of sympathetic nerve stimulation in mice

The right eyelid of the mouse contracted during cervical nerve stimulation thereby resembling the homologous structures of the other species. The frequency-response curve was flattened at the lower frequencies such that 5 Hz was needed for a 50% maximal response compared with about 2 Hz for a similar effect in rats and guinea-pigs (Table 1).

Stimulation of the right nerve caused tachycardia and the frequency-response curve (% maximum) lay between those constructed for rats and guinea-pigs. When measured in beats/min a greater tachycardia was seen in mice than in any other species, a maximum increase of 250 beats/min occurring in two of the mice when 20 Hz was used (Table 2).

Effects of sympathetic nerve stimulation in cats

Isometric contractions of the right nictitating membrane were recorded to stimulation of the intact cervical nerve; the threshold frequency was 0.5 Hz and larger responses occurred as the frequency was increased to 20 Hz, the highest employed, with which the contraction was well maintained in 2 out of 3 cats (Table 1).

By contrast with the other species the tachycardia to cervical nerve stimulation was small, indeed one cat failed to respond to frequencies lower than 20 Hz, and the left nerve was again less effective than the right (Table 2).

Mean blood pressure increased progressively in each animal as the frequency of stimulation was raised, the pressor response reaching a maximum of 40 to 60 mmHg. This effect was seen after nerve section only if the caudal ends of the severed nerves were stimulated, and was coincident with the tachycardia. Pulse pressure was rarely affected in these experiments.

Effects of vagal or sympathetic nerve stimulation in rabbits

In a similar manner to rats, the rabbits responded with a frequency-dependent bradycardia when the cardiac end of each sectioned vagus was stimulated and there was no significant difference between right and left vagi.

Fibres in the right sympathetic nerve were found to innervate both the ipsilateral nictitating membrane and inferior eyelid. Two experiments were performed on the membrane and two others on the eyelid instead as a means of establishing that the nerve being stimulated was the cervical sympathetic; since such a limited number of observations was made the results from each experiment are separately recorded in Table 1. Neither the inferior eyelid nor the nictitating membrane of the rabbit was able to maintain the response to 10 Hz stimulation which proved to be a more effective frequency than did 20 Hz.

Cardiovascular changes occurred on stimulating these nerves. The tachycardia never exceeded 65 beats/min and was not seen with frequencies lower than 1 Hz; recovery generally occurred within 60 seconds. Moreover, although stimulation of the left nerve produced slight increases in rate (about 40% of the maximal effect of right sympathetic nerve stimulation) the pulse pressure was mainly unaffected (Table 2). Small increases in blood pressure invariably occurred when each nerve was stimulated but these disappeared if, after nerve section, only the cephalic end was stimulated.

The action of some drugs on responses to sympathetic nerve stimulation

Most of the experiments in this section were performed on rats.

The adrenergic nature of the nerves innervating the heart and the eyelid was confirmed when the adrenergic neurone blocking agent guanethidine (3 mg/kg) reduced or abolished the effects on both structures of intermittent stimulation at 10 Hz, an inhibitory effect which was always preceded by sympathomimesis. Further support was obtained from the effects of α- and β-adrenoceptor blocking drugs, phentolamine (2.5 mg/kg) and propranolol (0.3 mg/kg) respectively. The former prevented the eyelid contraction but had no discernible effect on the tachycardia and the latter selectively abolished the cardiac response whilst slightly increasing the response of the eyelid.

The effects of hexamethonium were less reproducible. At all doses (2.5 to 25 mg/kg) responses to nerve stimulation of both the heart and eyelid were reduced, the latter more than the former. Raising the dose of hexamethonium from 10 mg/kg to 25 mg/kg produced no greater suppression of the tachycardia but the eyelid response was further blocked. In 4 experiments frequency-response curves were determined before and after 5 or 10 mg/kg hexamethonium; the responses to high frequency stimulation of the eyelid were more strongly inhibited than those to low frequency stimulation as is usually the case with ganglion blockade, but curiously the reverse was true with the cardioaccelerator response.

In 3 experiments atropine (1 mg/kg), following hexamethonium administration, further suppressed both cardiac and eyelid responses, the effect on heart rate being the more pronounced. Atropine given alone in two rats did not affect the eyelid responses but slightly reduced the tachycardia when either the right or the left nerve was stimulated at 10 Hz intermittently.

Two experiments were performed in the guinea-pig using hexamethonium 10 mg/kg; the drug depressed both the cardiac and eyelid responses to 10 Hz stimulation, the former by 30 and 50% the latter by 70 and 60% respectively in the two animals. In one of these experiments, the guinea-pig was further given atropine 1 mg/kg and the two drugs in combination almost completely obliterated the responses of both tissues. Four rabbits were given 5 mg/kg hexamethonium, and in each case the response of the eyelid to 10 Hz stimulation was reduced by a greater extent than that of the heart (a mean of 75% compared with 40%). In the one cat which received 5 mg/kg hexamethonium a complete suppression occurred of the tachycardia to 10 Hz stimulation whilst the nictating membrane response was reduced by 75%.

Whenever hexamethonium was given to animals where vasopressor responses were evident there was a dramatic reduction in the pressor effect regardless of the extent of cardiac or eyelid inhibition.

Discussion

Ganglion cells, in numbers running into thousands, are present along the length of the cervical sympathetic trunk and may be scattered or aggregated into visible ganglia (for diagram showing arrangements of pre- and post-ganglionic fibres from studies in cats and rabbits see Douglas, Lywood & Straub, 1960). It might therefore be anticipated that electrical stimulation of the intact cervical sympathetic nerve should evoke responses not only from structures lying clearly on the nervous pathways cephalad to the electrodes but also from organs more remote which are considered to receive their major innervation from other sources.

In the present study several species responded with cardioacceleration when the cervical sympathetic nerves were stimulated. The reproducibility of the cardiac responses among these animals, with the possible exception of the cat, suggests that it was not a case of aberrant sympathetic fibres being excited but, instead, a well-defined regular nervous pathway. It seems most surprising, in view of the countless investigations of the sympathetic nervous system over recent decades, that such a major pathway has not previously been described.

The first quantitative assessments in this study were made in rats. It soon became evident that tachycardia in excess of 150 beats/min could be regularly evoked with frequencies of 5 or 10 Hz applied either to the intact right sympathetic nerve or to the sectioned caudal end. The participation of afferent fibres in reflex activation of the heart was ruled out by the demonstration that tachycardia still occurred in rats whose spinal cord had been destroyed by orbital pithing.

The frequency-response curve of the heart to stimulation of the right nerve was changed after pithing. A more pronounced tachycardia occurred in the frequency range 0.1 to 0.5 Hz, and indeed responses of 80 and 90% maximal were observed with frequencies of 1 and 2 Hz respectively compared with the 2 and 5 Hz required in anaesthetized rats. It is conceivable that when the central nervous system remained intact either compensatory reflex mechanisms were generated by the cardioacceleration or else afferent fibres with an ultimate modulating function were being directly excited also. Whatever should be the explanation the results in the pithed rat preparation were remarkably similar to the graphs of cardioacceleration caused by stimulation of the spinal cord at the level C7-T1 with a pithing rod (Armstrong & Boura, 1973).

Both the latter authors and Gillespie, Maclaren & Pollock (1970) reported that little change in blood pressure accompanied the cardioacceleration which the localized spinal stimulation evoked. In the present investigations rises in blood pressure were often recorded, especially with the higher frequencies of stimulation. It is unlikely that these responses were simply generated by the primary cardiac effect, particularly since both diastolic and systolic pressures were elevated. Therefore vasoconstrictor fibres seem to exist in this nerve although their regional distribution is unknown.

It was not entirely unexpected that stimulation of the left nerve should produce a smaller tachycardia accompanied by a rise in pulse pressure. Furnival, Linden & Snow (1973) showed that, in dogs, stimulation of the left cardiac sympathetic nerves in the chest caused a smaller chronotropic but a greater inotropic effect than the nerves on the right. A number of references are cited in this paper which provide evidence of an uneven anatomical distribution of the left and right sympathetic fibres. Furthermore, Armstrong & Boura (1970) claimed that stimulation of the left sympathetic nerves of cats caused only positive inotropic effects whilst the nerves on the right produced tachycardia. It is therefore possible that in rats, guinea-pigs and rabbits cardiac efferent fibres running in the right cervical sympathetic nerve predominantly innervate the sino-atrial node whereas a less prominent nodal but a more obvious ventricular innervation is supplied by the left nerve. It should be emphasized that no conclusive evidence is yet available, since, as was indicated by Furnival *et al.* (1973), attempts must be made to control changes in parameters such as heart rate and blood pressure which could indirectly alter the inotropic state of the myocardium.

In a few experiments atropine was administered to discover whether vagal inhibitory fibres might have been simultaneously excited with the sympathetic nerves, thereby reducing the cardioacceleration which would have occurred by solely stimulating the sympathetic supply. This was particularly important during excitation of the left nerve which invariably produced smaller cardiac effects than its right counterpart. Atropine never potentiated the tachycardia, indeed a small reduction in the effect was the rule. This provides further support for a differential distribution of cardiac fibres in each nerve

There are species differences in the absolute values for maximum tachycardia; in mice the increase of 225 beats/min over resting levels of 520 beats/min was a change of 43%; in anaesthetized rats it was 60%, pithed rats 47%, guinea-pigs 47%, rabbits 14% and in cats 8%. No ready explanation is available for these differences although experiments are planned in which a variety of cardiac stimuli will be compared with the ability of the sympathetic nervous system to evoke responses to discover whether the limitations reside in the nervous system or the effector organ.

Stimulation of the intact cervical sympathetic nerve also resulted in tension changes of the ipsilateral inferior eyelid or the nictitating membrane. The reasonable surmise that mice and guinea-pigs would so respond, despite an absence of such investigations in the literature, was confirmed. Certain characteristics of response were common to all the species: the threshold frequency was about 1 Hz and maximum increases in tension occurred with 20 Hz; the muscles were seldom able to maintain their tension at this frequency. In none of the experiments did stimulation of the contralateral nerve produce contraction of the eyelid.

Histamine-induced rises in inflation pressure in the guinea-pig were reduced by concurrent stimulation of the right cervical sympathetic nerve. Daly & Mount (1951) reported bronchodilatation in the cat when the caudal end of the cervical sympathetic nerves were stimulated although Petrovskaia (1939) induced bronchoconstriction in guinea-pig isolated lungs with sympathetic stimulation, which was converted to bronchodilatation in the presence of ergotoxine. The present findings might be the result of stimulating sympathetic bronchodilator fibres but since the airway changes were accompanied by tachycardia and rises in blood pressure they could conceivably have been brought about indirectly.

Although there is little doubt that adrenergic nerves are ultimately responsible for both the cardiac and eyelid responses described in this paper the anatomical pathway taken by the cardiac fibres is not clear. Studies with hexamethonium alone and in combination with atropine suggest that the majority, if not all, of the stimulated sympathetic fibres were pre-ganglionic. It would appear reasonable on anatomical grounds that the fibres emerge from the upper thoracic region of the spinal cord and extend cephalad in the cervical nerve. Two explanations, advanced by Daly & Mount (1951) to explain their findings of bronchodilatation in the cat with cervical sympathetic stimulation, provide plausible alternatives for the subsequent pathways. The first assumes that the pre-ganglionic fibres loop near the superior cervical ganglion and then descend the nerve to synapse in, say, the stellate ganglion. The second involves a preganglionic axon reflex whereby fibres normally running up to the superior cervical ganglion branch at a lower level to make additional synaptic contacts with stellate ganglion cells. No evidence appears from the present studies to favour one explanation or the other.

The results in the present paper suggest that a major addition is available to the list of preparations *in vivo* for the study of sympathetically-innervated structures. A very simple dissection technique reveals nerves in the neck of several mammalian species whose stimulation produces effects both on accessible peri-orbital structures and on the heart. Thus the need for thoracotomy to expose paravertebral sympathetic ganglia for electrical stimulation is avoided and the analysis of drugs which modify sympathetic transmission is made relatively simple.

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(Received November 8, 1974.
Revised February 3, 1975)

Br. J. Pharmac. (1975), 54, 359-366

RENAL TUBULAR TRANSPORT OF PARACETAMOL AND ITS CONJUGATES IN THE DOG

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1 The renal tubular transport of paracetamol and its conjugates was investigated with renal clearance and stop flow studies in the dog. Paracetamol is sparingly bound to plasma proteins and therefore undergoes glomerular filtration. It is reabsorbed in the renal tubules by simple diffusion.

2 The conjugates of paracetamol, the sulphate and the glucuronide, both undergo glomerular filtration being weakly protein bound. At low concentrations in plasma both compounds are secreted by an active transport process. At higher concentrations both compounds are reabsorbed. Clearances are not dependent on urinary pH or flow rate. It is concluded that reabsorption is not a passive process but that there is an active bidirectional transport of the conjugates.

3 Net tubular secretion of the sulphate, but not the glucuronide, conjugate was inhibited by the administration of probenecid.

Introduction

The metabolism and toxicity of paracetamol have recently undergone intensive study for two reasons. First, the drug and its metabolites form approximately 80% of the metabolites of phenacetin (Brodie & Axelrod, 1949), a drug which has been strongly implicated in the pathogenesis of analgesic nephropathy (Dubach, Levy, Ehrensperger, Baumeler, Muller, Peier & Rosner, 1974). Secondly, paracetamol itself has gained considerable popularity within recent years as an antipyretic and analgesic and its wider availability has led to its use in attempted suicides with resultant hepatic necrosis (Clark, Thompson, Borirakchanyavat, Widdop, Davidson, Goulding & Williams, 1973).

Analgesic nephropathy is a unique type of renal injury in that the medulla is primarily affected and the cortical lesions appear secondary to medullary damage (Lindeneg, Fisher, Pedersen & Nissen, 1959). This implies that in order to produce damage either a drug or its metabolites must be selectively concentrated within the medulla, or that this region of the kidney has unique metabolic features which are affected by the drug, or that both factors are operative.

There have been a number of studies on the renal excretion of paracetamol (Barraclough, 1972; Prescott & Wright, 1973; Silberbusch, Lenstra, Leynse & Gerbrandy, 1974), but there have been no comprehensive studies on the mechanisms underlying the tubular transport of either the parent compound or its metabolites. In the present study we have investigated these problems with clearance and stop flow techniques.

Methods

Animal experiments

Sixteen female mongrel dogs were used, each weighing between 10 and 15 kg. Anaesthesia was induced with intravenous pentobarbitone 30 mg/kg and maintained with small intravenous doses as necessary. A midline lower abdominal incision was made and the ureters were cannulated at the level of the pelvic brim. Blood samples were taken from a femoral artery and blood pressure was monitored with an intra-arterial cannula in either a femoral or a carotid artery using 16 gauge polyethylene cannulae and a Sanborn 964 polygraph and pressure transducer. Intravenous infusions were administered with a Harvard peristaltic pump for larger volumes and a Braun syringe for smaller ones. In 10 dogs, paracetamol was given intravenously in an initial dose of 40 mg/kg followed by a 1% solution in isotonic saline at 4 mg/min, in 6 dogs the initial dose was 100 mg/kg followed by 10 mg/minute. Inulin was prepared as a 1.5% solution in isotonic saline to which 20 μCi of [^{14}C]-inulin (New England Nuclear) was added per 100 ml. After an initial injection of 20 ml, this was infused at 0.9 ml/minute. Urine for all clearance periods was collected for 15 min with a mid-point blood sample. Urine pH was recorded immediately with glass electrode and Leeds Northrup pH meter. A single experiment was done with the stop-flow technique according to the procedure previously presented in detail (Mudge, Cucchi, Platts, O'Connell & Berndt, 1968).

Due to the restriction of water intake for the preceding 18 h, most animals were hydropenic and excreted urine of high osmolality. To vary the rate of urine flow, mannitol was infused in a 5% solution. Urine pH was varied by administering 0.2 g/kg body weight of ammonium chloride in the diet for 1-2 days before the experiment and an additional 0.1 g/kg intravenously at the start of the experiment, subsequently followed by an infusion of 1% sodium bicarbonate (2 experiments) or the more rapid injection of a 10% solution (2 experiments). In those experiments in which the plasma concentration of paracetamol was varied, this was accomplished by increasing the infusion rate of the drug. Probenecid was administered intravenously in a dose of 40 mg/kg. All results are summarized as mean ± standard error of the mean. Statistical evaluations were made with Student's *t* test for unpaired data, regression analysis by the method of least squares and analysis of covariance (Snedecor, 1956). *P* values of less than 0.05 are regarded as significant. Clearance ratios refer to the clearance of a compound divided by the clearance of inulin.

Chemical methods

Paracetamol was estimated in duplicate by a modification of the method of Brodie & Axelrod (1949). A volume of plasma or urine containing 5-50 μg of paracetamol was diluted with distilled water to 2 ml. Sodium chloride (1 g) was added and the paracetamol was extracted into 20 ml diethyl ether U.S.P. grade, containing 1.5% iso-amyl alcohol. Of the ether phase 15 ml was transferred and the compound re-extracted into 3.2 ml of 0.1 N NaOH. Of the NaOH solution, 3 ml was then boiled for 30 min after the addition of 0.8 ml 12 N HCl in a 16 x 150 mm test-tube covered with a glass marble. The solution was then cooled, 0.1 ml 1% sodium nitrite was added, mixed, allowed to stand at room temperature for 20 min, following which 0.1 ml of 5% ammonium sulphamate was added, mixed, and allowed to stand for 3 minutes. Of a freshly prepared solution of 12% α-naphthol (Eastman Kodak) 0.1 ml was then added, shaken briefly, and then 2 ml of 6 N NaOH was immediately added, mixed on a Vortex Mixer for 3 s and then the tube was placed in crushed ice. Optical density was read on a Gilford spectrophotometer at 510 mu. Internal standards, reagent blank and biological blank were run simultaneously. Results with this procedure were similar to those of Brodie & Axelrod (1949) and Routh, Shane, Arredondo & Paul (1968), with the advantage that a larger number of samples could be processed at one time. The timing after the addition of the α-naphthol is critical as the dye formed is sensitive to temperature and time with deterioration after the times specified. Recoveries of paracetamol added to plasma and urine were 100 ± 1.58%. The maximum biological blank value was 1.2 μg/ml in urine and was always negligible in plasma. Eastman Kodak α-naphthol does not require sublimation but other suppliers' samples may be oxidized and hence inactive. Samples of diethyl ether need to be water saturated prior to use. If there is excess ethyl alcohol present in the particular batch then conjugates can be extracted and interfere with the estimation.

The conjugates of paracetamol (paracetamol-conj) were estimated in duplicate by incubating 0.01-1 ml of plasma or urine, with 1 ml of acetate buffer at pH 5 containing 5 mg/ml of β-glucuronidase and aryl sulphatase from *Helix pomatia* (Sigma type H 1) for 24-48 h at 37°C. The solution was then assayed for paracetamol as above, and paracetamol-conj calculated as the difference before and after enzymatic hydrolysis. The results were expressed as μg of paracetamol. Sample hydrolysis is complete in 16-24 h as determined by thin layer chromatography using the system outlined below.

Paracetamol sulphate (paracetamol-SO_4) and paracetamol glucuronide (paracetamol-gluc) were determined in duplicate on ultrafiltrates of 2-3 ml of plasma prepared by the method of Toribara, Terepka & Dewey (1957). Of the ultrafiltrate 200 μl was streaked on a silica gel (100 μm) thin layer chromatogram containing an ultraviolet phosphor (Eastman Kodak). Urine containing 50-100 μg of total paracetamol was chromatographed without pre-treatment. The chromatographs were developed using System III of Buch, Pfleger, Rummel, Ullrich, Hey & Staudinger (1967) consisting of acetone, butanol, water, 50 · 40 · 10. The sites of the conjugates were identified with ultraviolet light. In this system the R_F values were: paracetamol, 0.91; paracetamol-SO_4, 0.68; and paracetamol-gluc, 0.20. The area of silica gel containing the conjugate was then removed and added to 2 ml of β-glucuronidase and aryl sulphatase (as above), and the procedure completed as previously described. Internal standards and reagent blanks were carried through the

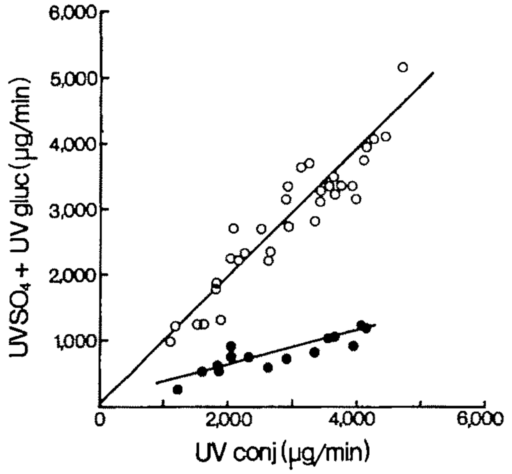

Figure 1 A comparison of two methods of determining paracetamol conjugates in urine. A regression analysis by the method of least squares (Snedecor, 1956) of the excretion rate of the total conjugates (o) vs the sum of the individual conjugates gave $y = 0.99x + 29.4$; $r = 0.926$ and the slope is significantly different from 0 at $P < 0.001$. Paracetamol-SO_4 (●) excretion rate alone (excluding studies with probenecid) showing the increase relative to the total conjugate.

entire procedure. Recovery of individual conjugates is complete when compared to the method for total conjugates (Figure 1). The plasma assay estimates the non-protein bound conjugate.

Inulin-[^{14}C] (New England Nuclear) was determined using 0.1-0.2 ml of plasma or urine with P.C.S. Solubilizer (Amersham-Searle) added to a final volume of 10 ml. This was counted in a Packard Tricarb 3310 Liquid Scintillation counter using external automatic standard for quench correction. Concentrated urine samples were diluted and alkaline urine samples were acidified to maintain stable counts. Counts remained stable over a 48 h period. In some experiments, inulin was determined by the anthrone method (Fuhr, Kaczmarczyk & Kruttgen, 1955).

Results

Protein binding

The amount of paracetamol bound to plasma protein was estimated by the method of Toribara *et al.* (1956), in ten samples from three dogs. The plasma concentration ranged from 24 µg/ml to 94 µg/ml. Binding was estimated to be 11.0% ± 1.2% and was independent of plasma concentration over the range studied. Paracetamol

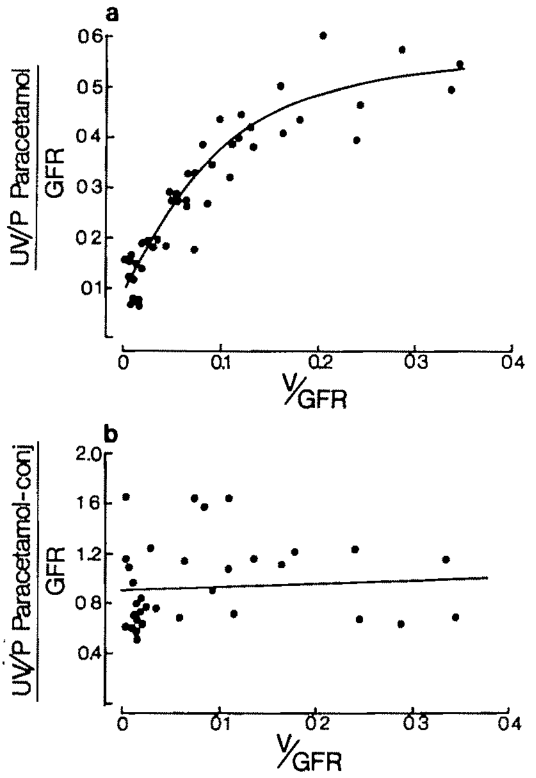

Figure 2 (a) Effect of fractional excretion of water on paracetamol clearance ratio. Summary of six experiments. Plasma paracetamol level was kept constant for each experiment with a range from 20 to 200 µg/ml for all animals. Line drawn by inspection. (b) Effect of fractional excretion of water on paracetamol-conj clearance ratio. Data from same experiments as Figure 2a. Plasma paracetamol-conj ranged from 12-70 µg/ml but was essentially constant for each experiment. $Y = 0.35x + 0.91$; $r = 0.2$. Slope is not significantly different from zero, $P = 0.50$.

clearances have been calculated without correction for plasma binding.

From the total paracetamol-conj estimated in plasma and the sum of the paracetamol-SO_4 and paracetamol-gluc estimated in plasma ultrafiltrate, the degree of binding is 13%. Clearances from the individual conjugates have been estimated on the basis of the observed concentrations in ultrafiltrates.

Effect of rate of urine flow

The clearance ratio of paracetamol increased with the fractional excretion of water (Figure 2a) but the paracetamol-conj ratio remained unchanged (Figure 2b). The individual conjugates (paracetamol-SO_4 and paracetamol-gluc) showed no

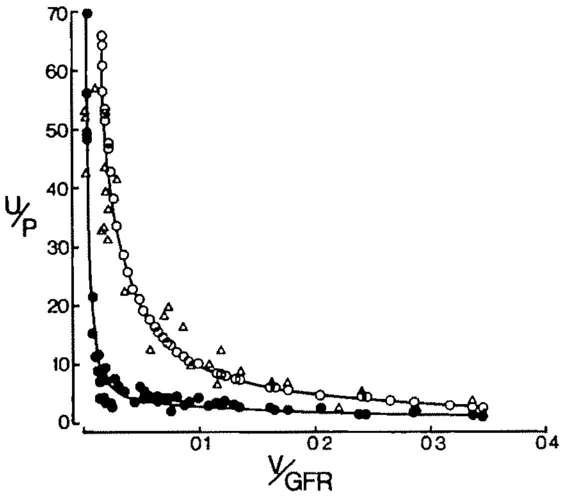

Figure 3 Urine/plasma concentration ratio for paracetamol, paracetamol-conj and inulin plotted against the fractional excretion of water. Lines are drawn by inspection; (●) paracetamol; (△) paracetamol-conj; (○) inulin (lines for inulin and paracetamol-conj are co-incidental).

significant change in their clearances as a result of the rate of urine flow. From Figure 3, the urine/plasma (U/P) concentration ratio of paracetamol is highest at the lowest rates of urine flow. The U/P ratio falls dramatically with a slight increase in the fractional excretion of water. The U/P ratios for inulin and paracetamol-conj fall less steeply. For paracetamol, the highest U/P ratio occurs at the lowest clearance.

Effect of urine pH

The clearance ratio of paracetamol remained unchanged over the entire range of urine pH values. In Figure 4a this ratio is shown as a function of the fractional excretion of water with regression estimates for three groups of pH values. The slight slope of the line is a result of a small diuresis from sodium bicarbonate infusion. The increase in clearance is as would be predicted from a change in urine flow rate alone. Clearance ratios of paracetamol conjugates (Figure 4b) did not vary with urine pH. Individual conjugates were estimated in two dogs over ten clearance periods. Paracetamol-SO_4 between pH 5 and 6 had a mean clearance ratio of 1.03 ± 0.14 and between 7 and 8 of 1.37 ± 0.10 ($P = 0.21$). For the same pH ranges paracetamol-gluc clearance ratios were 0.77 ± 0.04 and 0.75 ± 0.04 respectively ($P = 0.89$).

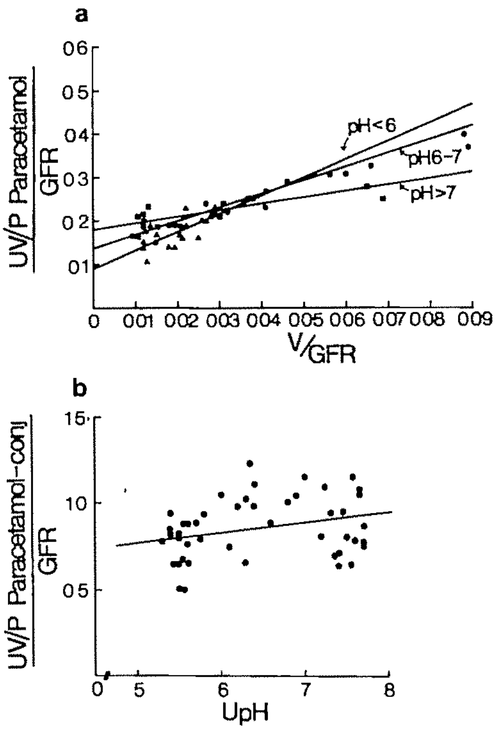

Figure 4 (a) Effect of urine pH on clearance ratio of paracetamol. Results of four experiments in which a small increase in urine flow rate occurred as a result of bicarbonate administration. At pH > 7 (○) $y = 3.853x + 0.099$, $r = 0.68$; for pH < 6 (△) $y = 2.83x + 0.139$, $r = 0.97$. By analysis of covariance these slopes are not significantly different ($P = 0.56$) from each other, but are significantly different from zero $P = 0.002$ and $P < 0.001$, respectively. At pH 6-7, slope is not different from pH < 6 or pH > 7. (b) Effect of urine pH on the clearance ratio of paracetamol-conj. Same experiments as above. $y = 0.57x + 0.49$, $r = 0.28$. The slope is not significantly different from zero ($P = 0.06$).

Effect of plasma level on drug

The clearance ratio of paracetamol remained unchanged over a wide range of plasma drug concentration in two dogs, and in a third there was a minimal increase in clearance at the higher drug concentrations (Figure 5a). The difference in the clearance ratios in these three dogs is due to the difference in the spontaneous rates of urine flow. Taking the results from all three animals together, the plasma concentration of paracetamol had no significant effect on its clearance. However, the clearance ratio of paracetamol-conj showed a significant decrease with increasing plasma concentrations (Figure 5b). The same phenomenon is seen when the individual conjugates are measured separately (Figures 5c and 5d).

Effect of probenecid

This agent was given in a dose known to inhibit the renal tubular transport of many organic acids (Weiner, 1973). It had no effect on the clearance ratio of paracetamol, paracetamol-conj, or paracetamol-gluc (Table 1). However, probenecid significantly decreased the paracetamol-SO_4 clearance ratio, the mean fall being 44% of the control.

Stop-flow study

A single stop-flow experiment was undertaken to define the tubular localization of the transport of paracetamol and its conjugates and to examine certain mechanisms of transport (see discussion section) suggested by the results from conventional clearances. As previously reported with the identical stop-flow technique (Mudge *et al.*, 1968) the static column of fluid which had remained in contact with the distal nephron is represented by the nadir of urinary chloride concentration (samples 4-8, Figure 6), while urine previously in contact with the proximal tubule is represented by sample numbers about twice that great. The results shown in Figure 6 indicate that the reabsorption of paracetamol is accentuated in the distal area and that this is unaffected by probenecid. Paracetamol-SO_4 undergoes net proximal secretion which is probenecid-sensitive. With paracetamol-gluc there is net reabsorption without direct evidence of its tubular localization and with no apparent effect of probenecid.

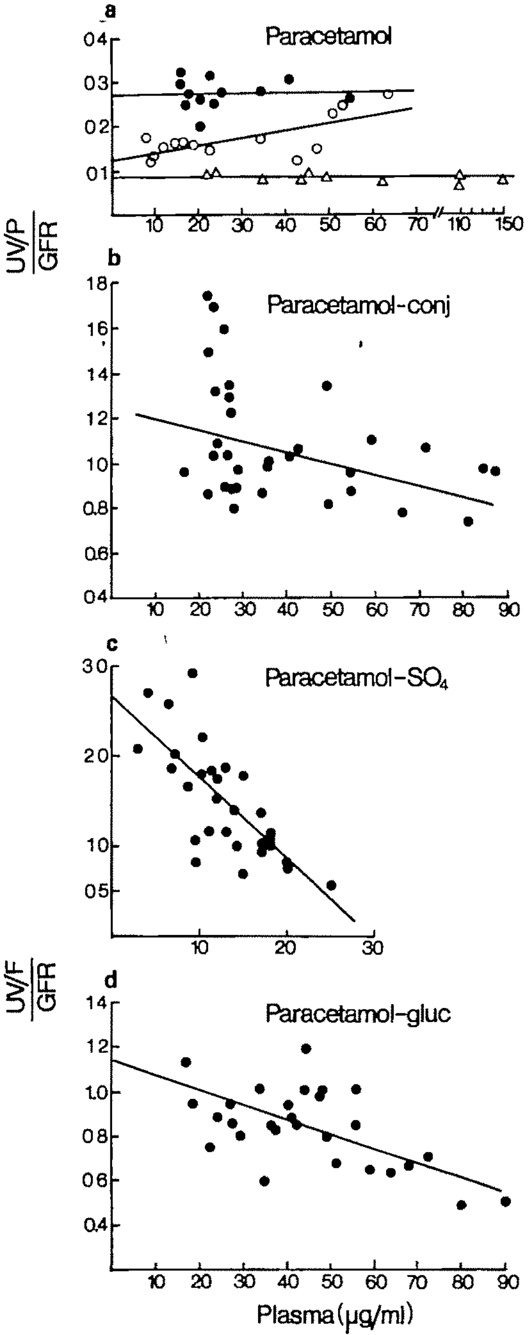

Figure 5 (a) Effect of plasma drug concentration on clearance ratio of paracetamol. Results from three experiments in which rate of urine flow was constant for each but differed from one to another, (●) > (○) > (△). Urine pH ranged from 6 to 7. Slope is not significantly different from zero for (●) and (△), but is different for (○) ($r = 0.694$, $P = 0.006$). For all three experiments together, over the comparable plasma range from 10-70 µg/ml, the slope is not significantly different from zero ($P = 0.68$). (b) Effect of drug plasma level on clearance ratio of paracetamol-conj. Same animals as Figure 4a. By regression analysis, $y = 1.259 - 0.004x$, $r = -0.46$, $P = 0.006$. (c) Effect of drug concentration of plasma ultrafiltrate on clearance ratio of paracetamol-SO_4. Summary of all data from seven experiments in each of which the rate of urine flow and urine pH were essentially constant. By regression analysis, $y = 2.66 - 0.09x$, $r = -0.71$, $P < 0.001$. (d) Effect of drug concentration of plasma ultrafiltrate on clearance ratio of paracetamol-gluc. Same experiments as Figure 4c. By regression analysis, $y = 1.158 - 0.007x$, $r = -0.62$, $P < 0.001$.

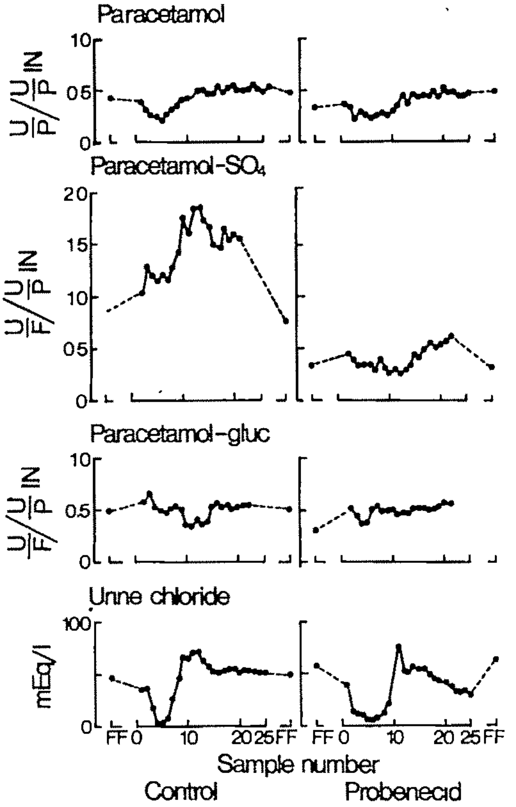

Figure 6 Stop-flow analysis of tubular transport of paracetamol and its conjugates, before and after probenecid. Flow was stopped for 6 min following which urine collections were 0.6 ml each. FF = free-flow. Probenecid given intravenously in dose of 40 mg/kg. Plasma drug concentrations rose between first and second stop-flow procedures; the range for the entire experiment was paracetamol 88-142, paracetamol-SO_4 20-39, and paracetamol-gluc 80-145 μg/ml. Control U/P inulin ratios were 5 and 8.

Discussion

The renal excretory mechanisms for paracetamol appear to involve filtration and reabsorption by passive diffusion of the non-ionic form. The relevant evidence includes the following: 1. paracetamol/inulin clearance ratios far less than unity; 2. the lipid solubility of paracetamol; 3. the curvi-linear positive correlation between clearance and the rate of urine flow; 4. the fact that clearance is independent of the plasma concentration and hence also of the amount reabsorbed; and 5. the absence of any effect of probenecid. Since paracetamol is a weak acid with a pKa of 9.5 (Prescott & Wright, 1973) one would not anticipate that its clearance would be influenced by changes in urinary pH within the achievable physiological range (Weiner, 1973). Stop-flow analysis failed to localize a unique reabsorptive site and reabsorption throughout the entire nephron may be inferred. The above interpretation would not be changed if the data were corrected for the slight degree of binding to plasma proteins. Thus the excretory pattern of paracetamol is somewhat similar to that of urea and its analogues.

The excretory mechanisms for the conjugates are different from those of the parent compound, and the excretory pattern of sulphate and glucuronide conjugates are somewhat different from each other. For both, clearance is not affected by urine pH or the rate of urine flow, but is strongly influenced by the concentration of the conjugate in the plasma (or ultrafiltrate). Clearance, corrected for plasma binding, shows net tubular secretion at low plasma levels and net reabsorption at high. Thus, each conjugate undergoes bidirectional tubular transport. The secretory and reabsorptive mechanisms may be considered separately. For paracetamol-SO_4 the relationship between net secretion and the plasma levels suggests that secretion is a rate limited

Table 1 Effect of probenecid on the clearance ratio (clearance/G.F.R.) of paracetamol and its conjugates

Compound	*Plasma conc. range (μg/ml)*	*Mean clearance ratio Control*	*Mean clearance ratio Probenecid*	P*
Paracetamol	74-142	0.234 ± 0.05	0.227 ± 0.04	0.92 NS
Paracetamol-conj	64-121	0.79 ± 0.05	0.725 ± 0.03	0.65 NS
Paracetamol-SO_4	9-39	1.18 ± 0.15	0.57 ± 0.016	0.005
Paracetamol-gluc	18-145	0.78 ± 0.08	0.81 ± 0.13	0.84 NS

* P = significance probability from Student's t test on unpaired data.
NS = not significant.
Values are based on a total of 18 clearance periods from three dog experiments.

process. By stop-flow analysis this transport system is in the proximal nephron. It is also probenecid-sensitive. Paracetamol-SO_4 secretion thus shares many features in common with other organic acids (Weiner, 1973). Paracetamol-gluc behaves in a similar manner. However, its secretion appears not to be inhibited by probenecid, nor has the tubular site of secretion been positively identified. In the presence of bidirectional transport, the lack of an effect of probenecid could be due to an insensitivity of the secretory mechanism to this agent or to approximately equal sensitivities of both the secretory and reabsorptive mechanisms. Precedent for drug inhibition of transport in both directions has been provided in the case of uric acid transport (Weiner & Tinker, 1972). The present data do not permit one to distinguish between the above possibilities. It should be emphasized that at all doses of paracetamol the concentration of paracetamol-gluc is approximately three times greater than that of paracetamol-SO_4 in both plasma and urine. Therefore, under all conditions each conjugate is undergoing active secretion in the presence of the other. The degree to which this might result in competitive inhibition of secretion can not be evaluated.

Prescott & Wright (1973) reported that the renal clearance of paracetamol in man depended on the rate of urine flow but was unrelated to urine pH, and that the clearance of the conjugates was independent of both flow rate and pH. The renal mechanisms in the dog thus appear to be similar to those in man.

Non-ionic diffusion is the most common mechanism regulating the renal tubular reabsorption of drugs and their metabolites (Weiner, 1973). The sulphate and glucuronide conjugates of paracetamol have not been characterized as to their pKa or solubility in different lipid-like solvents. For both, lipid solubility less than the parent compound is apparent from the analytical method. It may be presumed that, like other glucuronides, the pKa of paracetamol-gluc is in a range in which a change in urinary pH would effect excretion if the glucuronide were sufficiently lipid soluble (Weiner, 1973). In the case of paracetamol-SO_4, by analogy to other sulphate conjugates, the probable pKa may be so low that excretion would not be pH-dependent (Weiner, 1973). Since both conjugates appear relatively lipid insoluble and since for neither is excretion pH-dependent, it is highly unlikely that reabsorption occurs by non-ionic diffusion. Thus, both conjugates resemble *m*-hydroxybenzoate for which carrier mediated reabsorption and secretion have been demonstrated (May & Weiner, 1970).

The total conjugates are equal to the sum of the two individual conjugates. Since the glucuronide forms approximately 70% of the total, the behaviour of the total conjugates more closely resembles that of the glucuronide rather than that of the sulphate. This would explain the apparent absence of any effect of probenecid on total conjugates.

The tubular transport of these compounds has obvious implications for the pathogenesis of analgesic nephropathy. Paracetamol itself is relatively lipid soluble and undergoes reabsorption throughout the nephron. The conjugates, on the other hand, are probably transported in the proximal tubule. This suggests the possibility that paracetamol might be reabsorbed in the medulla, particularly in the collecting duct, at a high concentration. Previous work has suggested that N-hydroxy metabolites may be cytotoxic (Weisburger & Weisburger, 1973). Oxidative reactions are known to occur within the kidney in the case of phenacetin (Uehleke & Schnitger, 1969), but have not been studied for paracetamol. In the rat it has been shown that dehydration is a necessary prerequisite for the development of papillary necrosis (Saker & Kincaid-Smith, 1969).

In their entirety, the above relationships suggest that the following sequence of events may underly this type of nephropathy. Oliguria leads to maximal concentrations of paracetamol within the tubular fluid of the collecting duct. The reabsorption of paracetamol at this site might lead to a high intracellular level which, as the substrate for the oxidative reaction, would enhance the formation of the more cytotoxic metabolites. Critical to this mechanism is the concentration of paracetamol within the tissue of the medulla. Bluemle & Goldberg (1968) have previously proposed that paracetamol is concentrated within the medulla. However, their data fail to distinguish between the drug within the distal tubular fluid and that which might be present either within the cells or in the interstitial fluid. Although both paracetamol and its conjugates may give rise to high medullary concentrations as determined by analysis of whole tissue, from a consideration of the mechanisms of their transport it is unlikely that they are distributed in a similar manner between the several tissue compartments. Additional experiments are in progress in an attempt to provide a definitive answer. The mechanisms by which phenacetin is transported by the renal tubule are also under study.

This study was supported by National Institutes of Health Grants AM 06818-13 and AM 16960-01. G.G.D. is a Fellow of the Postgraduate Medical Foundation of the University of Sydney. We are indebted to L. Akerman for his technical assistance.

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(Received March 3, 1975
Revised April 4, 1975)

Br. J. Pharmac. (1975), 54, 367-374

DECAMETHONIUM IN THE PERFUSED AND IMMERSED RAT DIAPHRAGM

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1 The water content and mannitol space of rat diaphragms which were perfused through the inferior vena cava was increased compared with immersed diaphragms. The potassium content of both preparations, when expressed in terms of dry weight, was maintained at similar values to that found *in vivo*.

2 Despite the application of a constant concentration of decamethonium, a steady level of neuromuscular block was not obtained in either the perfused or immersed rat diaphragm. The immersed preparation differed from the perfused preparation in that recovery from paralysis occurred despite the continued presence of the drug.

3 The rate of uptake of labelled decamethonium (100 μM) at the end-plate region was similar in the perfused and immersed diaphragm. The slopes of the regressions were 0.059 and 0.054 μl mg^{-1}min^{-1} (based on dry weight) respectively which were not significantly different. This implies that the rate of uptake of the drug at the end-plate is slow and limited by the rate of entry into the fibre rather than by diffusion to the site of entry.

Introduction

Creese & Maclagan (1970) have obtained autoradiographic evidence that decamethonium enters muscle fibres in the rat. It has been suggested that the permeability of this organic ion at the end-plate is low and similar to that of sodium (Creese & England, 1970). If this is the case, then the uptake of decamethonium would be expected to be limited by entry at the cell membrane rather than by diffusion. If however, the permeability of decamethonium approached that of potassium then the uptake would be faster in a perfused muscle than in an immersed muscle because of delays due to diffusion in the latter (Klaus, Lullman & Muscholl, 1960; Keynes, 1954).

In the present study, rat diaphragms were perfused *in vitro* via the inferior vena cava with labelled drug to compare the rate of uptake with that in immersed muscles. In addition, the neuromuscular block produced by decamethonium was recorded in an attempt to confirm previous claims that a steady paralysis can be obtained with depolarizing drugs (Maclagan, 1962; Gibberd, 1966) when applied via the capillaries. The water, potassium and sodium content and also the mannitol space were determined in both preparations.

Methods

The composition of the saline was (mM): K^+5.0, Na^+145.0, Ca^{2+}1.3, Mg^{2+}1.2, Cl^-125.0, HCO_3^-25.0, $H_2PO_4^-$ 1.2, SO_4^{2-}1.2 (Creese & Northover, 1961). The glucose content was 200 mg/100 ml and the solution was gassed with 5% v/v carbon dioxide and 95% oxygen. The temperature was maintained at 38°C. Albino Wistar rats of 80-140 g were used. The animals were stunned and then decapitated.

For the recording of isometric muscle contractions, a strain guage (Statham micro-scale. model UL5) and a Statham transducing cell (model UC3) were connected to a pen recorder (Devices single-channel). The left phrenic nerve was stimulated with rectangular maximal pulses of 0.2 ms duration delivered at a frequency of 6/minute.

Immersed diaphragm

The left anterior portion of the diaphragm together with its rib was quickly removed, with or without the left phrenic nerve and transferred to a bath (100 ml) for recording of contractions, or to a tube (10 ml) for measurement of uptake of labelled decamethonium. Twitch tension was recorded by means of a strain gauge connected to the tendon by a thin platinum wire (0.4 g). The uptake of the labelled drug was measured in the manner described by Creese & England (1970). After immersion in labelled drug the muscle was

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washed in saline for 10 min, attached to a metal strip and frozen on solid carbon dioxide.

Perfused diaphragm

The preparation is similar to that described by Gibberd (1966). The inferior vena cava was cannulated through the right auricle and the entire diaphragm with the left phrenic nerve was transferred to cold saline. The abdominal inferior vena cava was then tied below the diaphragm and the liver dissected to leave the tied stump of the vein. The diaphragm was then transferred to the perfusion chamber (200 ml) where the atmosphere was kept moist by a thin aerated layer of saline at the bottom. The diaphragm was perfused by means of a roller pump (Watson-Marlow flow inducer) at a rate between 3 and 4 ml/minute. The muscle could be perfused from one of two jacketed reservoirs. The change-over time (i.e. the time taken for the second perfusion of saline to reach the tip of the cannula) was 6 s at a flow rate of 3.2 ml/minute. Contractions of the left side of the diaphragm were recorded by connecting the tendon to a strain gauge by means of a platinum wire (0.4 g). For uptake studies, the entire diaphragm was perfused first with saline and then switched to the reservoir containing saline plus labelled decamethonium. After a known interval the muscle was again perfused for 10 min with saline and the perfusion chamber was filled so that the diaphragm was immersed. The immersion fluid was changed after 5 minutes. The left anterior strip was then quickly dissected and frozen.

Freezing of diaphragms

The muscles were frozen on solid carbon dioxide in the manner described by England (1970) and sliced horizontally from tendon to rib in a direction parallel to the band of end-plates to produce a series of strips 1 mm wide. The frozen strips were rapidly weighted, transferred to polyethylene vials, dissolved and counted by scintillation methods (Creese & Taylor, 1967).

Analysis of muscles

Sodium, potassium and water content of muscles together with the mannitol space were measured by the method of Creese (1968).

Scintillation counting

Samples were counted in polyethylene vials at 6° C in a Packard 3000 Series Tri-carb Liquid Scintillation Spectrometer. The background was approximately 20 ct/min and the efficiency was 27.1% when estimated by a standard, tritiated *n*-hexadecane. The quenching produced by 6 mg of muscle tissue was less than 1%, and the counting rate was proportional to the radioactivity added to the vials.

Drugs used

Decamethonium dibromide (Syncurine, Burroughs Wellcome & Co., U.S.A.) mol.wt. 419; D(+)-mannitol (Hopkin & Williams Ltd., Chadwell Heath, Essex) mol.wt. 182; [^{3}H-methyl]-decamethonium dichloride (mol.wt. 329) with a specific activity of 1.1 Ci/mmol was obtained from the Radiochemical Centre, Amersham, Buckinghamshire. The compound was analysed at Amersham by thin-layer chromatography on alumina in chloroform-methanol (80:20 v/v) and the radiochemical purity was 98%.

Tritiated (+)-mannitol (mol.wt. 182) with a specific activity of 0.5 Ci/mmol was also obtained from the Radiochemical Centre where analysis by paper chromatography showed that the radiochemical purity was greater than 98%.

Results

Water and ion content

Table 1 shows an analysis of the water and ion content of rat diaphragms after immersion for 1 h and after perfusion for 1 h with saline. Although the whole diaphragm was perfused, only the left anterior portion was analysed.

As can be seen from Table 1 there is a small increase in the water content of the immersed muscle compared with the controls *in vivo*, similar to that obtained by Creese (1954) and Creese, El-Shafie & Vrbova (1968). There is a large increase in the water content of perfused muscles as compared with controls *in vivo*. The mannitol space is also markedly increased in the perfused muscle compared to the immersed muscle.

The potassium content (K^+/g wet tissue) is lower in the perfused preparation. This result is due to the high mannitol space, for when calculated on a dry weight basis the potassium content in immersed and perfused muscles shows little change when compared with controls *in vivo* (Table 1).

There is a large rise in the sodium content of the perfused diaphragm compared to the immersed preparation. From Table 1 it can be calculated that the increase in the mannitol space for the perfused diaphragm compared to the immersed diaphragm is 214 ml/100 g dried muscle. The sodium associated with this increased mannitol space is 31.0 mEquiv/100 g dried muscle. The large increase in the sodium content of the perfused preparation could therefore be attributed to the increase in the extracellular fluid. The mannitol space and the sodium content were not estimated on the same piece of muscle and for this reason it is not possible to obtain a valid estimate of the fibre sodium whose estimation involves a small difference between two large values (see Creese, 1968).

Neuromuscular block

Both immersed and perfused preparations were allowed to contract for 30 min or more to ensure that the contraction height was steady before the application of drug. After perfusion with saline the solution was changed so that the muscle was perfused with saline containing a known concentration of decamethonium.

Figure 1 shows a recording of such an experiment after perfusion with decamethonium, 150 µM. The block at this concentration commenced almost immediately and 100% block was achieved within 250-350 seconds. The paralysis could be quickly reversed by switching back to the saline reservoir, full recovery took about 250 seconds. Tachyphylaxis was not evident in the perfused rat diaphragm as can be seen from the figure. All the muscles used maintained full contractility during the experimental period which in some cases was 5 h or more.

The effect of smaller concentrations of decamethonium was also investigated. Concentrations of 45 µM and 10 µM decamethonium were infused from the second reservoir. The results obtained are illustrated graphically in Figure 2. With perfusions of 10 µM decamethonium there appears to be some potentiation of the twitch height which gradually wanes. There is later a small degree of block and it would seem possible that under the conditions of perfusion even small concentrations of decamethonium might eventually cause complete block. At no concentration was it possible to obtain a steady paralysis, and the block proceeded steadily to 100% with time. On washing out the drug, recovery took place quite rapidly in all cases (usually about 3-5 min), often with potentiation of the twitch height. In experiments with the immersed diaphragm it was found that partial recovery of the block occurred despite the presence of the drug, providing the dissection had been carried out sufficiently quickly. The recovery phenomenon is illustrated in Figure 3 with a concentration of 150 µM decamethonium. After washing out, full recovery occurred after 5 or more min with the immersed diaphragm, and as with the perfused diaphragm, there was some potentiation of the original twitch height. Again, tachyphylaxis was not evident.

Uptake of decamethonium at the end-plate

The histogram in Figure 4a represents the uptake of decamethonium in a rat diaphragm immersed in saline. The muscle was exposed for 20 min to the

Table 1 Water, sodium and potassium content and mannitol space of the immersed and the perfused rat diaphragm

Preparation	*Water content (mg/g)*	*Sodium content (m.equiv/ Kg wet wt)*	*Sodium content (m.equiv/ 100g dry wt)*	*Potassium content (m.equiv/ Kg wet wt)*	*Potassium content (m.equiv/ 100g dry wt)*	*Mannitol space (ml g^{-1})*
In vivo	769 (21) ± 14.3	33.9 (13) ± 5.8	14.4 (13) ± 2.3	95.2 (20) ± 5.9	41.2 (20) ± 3.4	—
Immersed (1 h)	789 (13) ± 14.7	44.6 (13) ± 9.9	21.2 (13) ± 4.6	84.0 (13) ± 8.1	39.8 (13) ± 2.5	0.298 (6) ± 0.063
Perfused (1 h)	837 (10) ± 11.1	70.6 (10) ± 5.8	43.5 (10) ± 3.8	67.9 (10) ± 4.3	41.8 (10) ± 1.8	0.578 (10) ± 0.141

Mean values are given ± s.d.
The number of estimates are shown in parentheses
Each muscle was maintained in saline by perfusion or immersion for 1 hour.

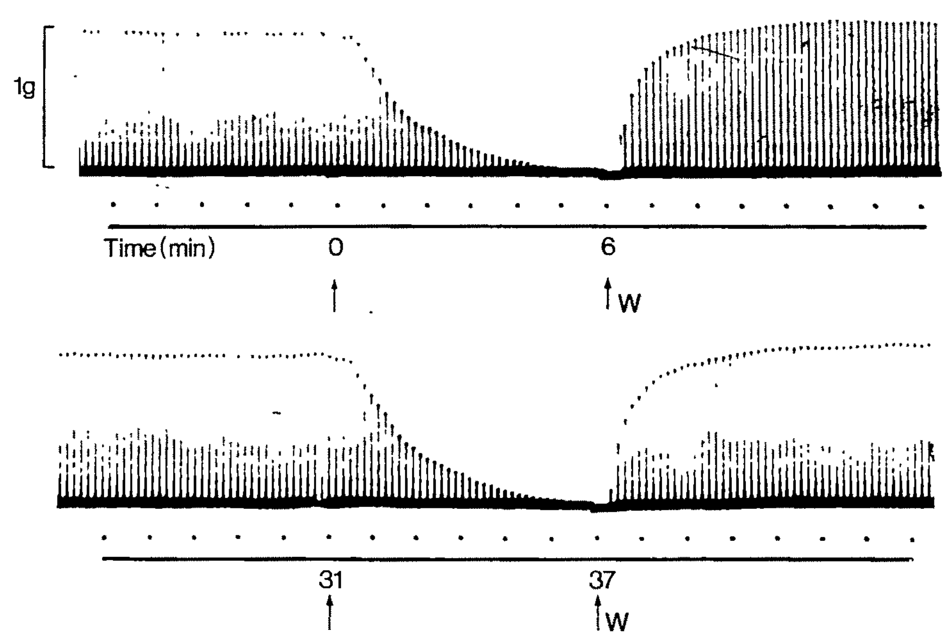

Figure 1 Rat 96 g Record of isometric maximal contractions of the perfused rat diaphragm *in vitro* elicited by stimulation of the left phrenic nerve at 6/minute. At the arrow (time = 0) saline containing 150 μM decamethonium reached the tip of the perfusion cannula by perfusion from a second perfusion reservoir (see text). At W the muscle was again perfused with physiological saline Thirty-one min after the first application the muscle was again perfused with 150 μM decamethonium (lower section). No tachyphylaxis was evident. A constant flow of 3.3 ml/min was used. Resting tension was equivalent to 5.3 g. The twitch tension produced is low since contraction of the left hemidiaphragm is recorded from the central tendon of the whole muscle.

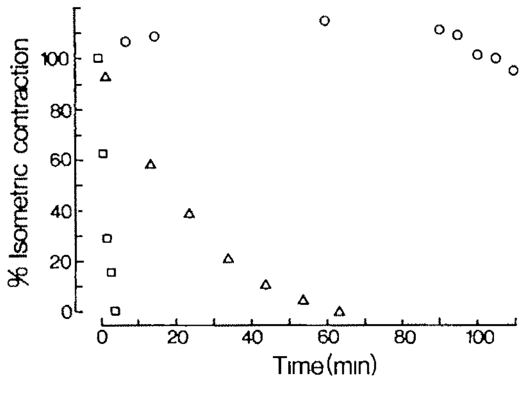

Figure 2 Graph to illustrate the effect of perfusion of the rat diaphragm with various concentrations of decamethonium. Ordinates represent the isometric maximal twitch tension expressed as a percentage of the control value. Abscissae represent the time of application of the drug. (□) 150 μM decamethonium, (△) 45 μM decamethonium, (○) 10 μM decamethonium. After changing back to perfusion with saline containing no decamethonium the muscle quickly recovered in all cases (see text).

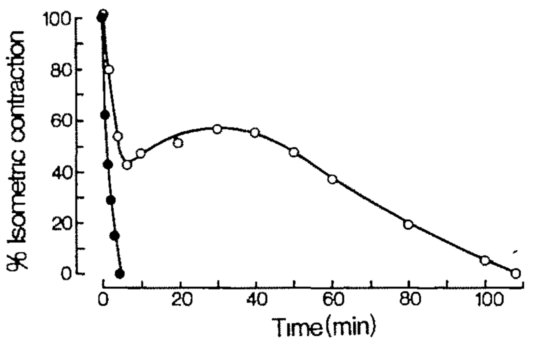

Figure 3 Comparison of effect of 150 μM decamethonium in the immersed and perfused rat diaphragm. Ordinates represent the percentage of the original maximal twitch. Abscissae give the time of application of the drug (●) perfused diaphragm 150 μM decamethonium, (○) immersed diaphragm 150 μM decamethonium. Note the recovery phenomenon evident in the immersed muscle, but not in the perfused preparation.

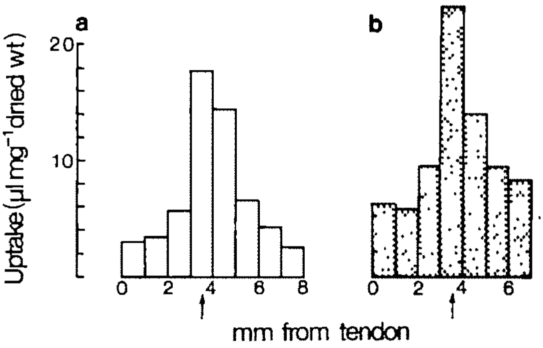

Figure 4 Uptake of labelled decamethonium in diaphragm of rat (20 min) in μl mg^{-1} dried weight. Concentration of 100 μM, with washout in inactive saline for 10 minutes. The arrows indicate the slice of muscle which contained the line of end-plates (see text). (a) Histogram with clear area shows uptake in an immersed diaphragm. (b) Histogram with stippled area shows uptake in a perfused diaphragm.

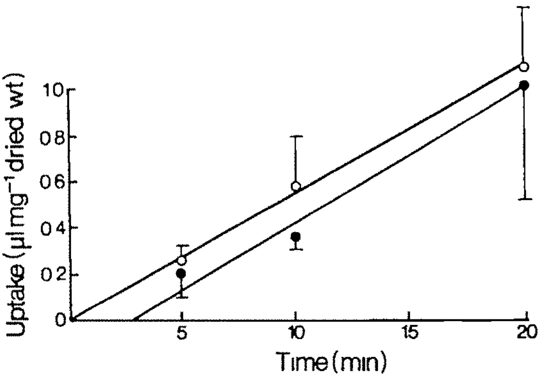

Figure 5 Specific uptake of labelled decamethonium (μl mg^{-1} dried wt.) at the end-plate region in rat diaphragms after immersion with 100 μM decamethonium (o) and after perfusion with 100 μM decamethonium (•) at various times. Each point represents the mean of at least four muscles and the limits give ± s.d. The rate of uptake is not significantly different in the two preparations (see text).

presence of labelled decamethonium (100 μM) and then washed with saline for 10 minutes. The uptake is expressed as clearance in μl mg^{-1} or (radioactivity per mg dried muscle)/(radioactivity per μl saline). Figure 4 shows the distribution of radioactivity along the muscle from tendon to rib, and the arrow indicates the slice which contained the line of end-plates, as shown by the white line which is seen when the muscle was frozen before being sectioned (England, 1970). The maximum radioactivity was at the end-plate, with a smaller uptake at the end of the fibre. This effect is similar to that described by Taylor, Creese, Nedergaard & Case (1965) and Creese & England (1970).

Table 2 Uptake of ^{3}H-decamethonium in the perfused and immersed rat diaphragm at various times

Preparation	*No.*	*Time (min)*	*Asymptotic uptake (µl mg.⁻¹ dried wt)*	*Specific uptake at end-plate (µl mg⁻¹ dried wt)*
	4	5	0.10 ± 0.01	0.26 ± 0.03
Immersed	4	10	0.22 ± 0.01	0.57 ± 0.11
	4	20	0.32 ± 0.01	1.08 ± 0.13
	5	5	0.20 ± 0.01†	0.20 ± 0.05
Perfused	5	10	0.32 ± 0.03*	0.36 ± 0.03
	5	20	0.71 ± 0.04†	1.01 ± 0.22

Mean values are given ± s.e.

* Significantly different from immersed diaphragm $P < 0.05$

† Significantly different from immersed diaphragm $P < 0.001$

The specific uptake on the end-plate region was obtained by subtracting the non-specific asymptotic uptake at the ends of the fibres from the total peak uptake at the end-plate region. See text.

Figure 4b represents the uptake of decamethonium in a diaphragm perfused for 20 min with saline containing labelled decamethonium (100 μM) and then washed for 10 min by perfusion with saline. The distribution of radioactivity along the muscle is qualitatively similar to that in the immersed muscle except that the uptake is somewhat larger. The non-junctional uptake is significantly larger in the perfused muscle at all times (Table 2). The uptake at the ends of the fibres (when expressed as a clearance) is independent of concentration and is probably attributable to diffusion (Creese & England, 1970). The significance of this increase in the non-specific uptake in the non-junctional region for the perfused diaphragm remains to be determined. In contrast, the peak uptake at the end-plate region (when expressed as a clearance) is concentration-sensitive and saturable (Creese & England, 1970; Humphrey, 1970) and is not significantly different in either the perfused or immersed diaphragm (Table 2).

Figure 5 illustrates this specific component of decamethonium uptake at the end-plate, which is obtained by subtracting the non-junctional value from the peak. The results from 15 perfused and 12 immersed muscles were expressed as μl mg^{-1} (dry weight) and plotted against time. The slopes of the simple regressions (Figure 5) are 0.0541 and 0.0586 μl mg^{-1} (dry weight) for the immersed and perfused muscles respectively. The difference between the slopes is small and it was concluded that the deviation from parallelism were not significant ($0.6 > P > 0.5$). The combined slope is 0.0566 (95% confidence limits 0.0429 and 0.0703). In Figure 5 the variance increases with time and hence weighted regressions were also calculated with a weighting factor equal to $^1/Y^2$ where Y is the expected value. The slopes of these regressions after three iterations were 0.057 and 0.048 μl mg^{-1} (dry weight) for the immersed and perfused muscle respectively, and again, the values of the slopes were not significantly different ($0.3 > P > 0.2$).

The simple regression for the immersed muscle has a small positive origin at 0.004 ml min^{-1}, while the simple regression for the perfused muscle has a negative origin at 0.16 ml min^{-1}. This indicates an apparent lag in the peak uptake of the perfused diaphragm which could be, in part, attributable to the increase in the extracellular space. However, the origins of the two regressions are not significantly different at the 5% probability level. In control experiments the perfusate was collected over 15 s intervals for 10 min during perfusion. It was found that over 90% equilibration occurred within 2 min with a half time of 32 s and no lag phase was evident.

Discussion

The rat diaphragm can be maintained by immersion so that the loss of intracellular potassium is small with only a moderate rise in the sodium content (Creese & Northover, 1961). In this study, the potassium content of the perfused muscle (K^+/g wet tissue) is low, but this is to be expected considering the high water content of the tissue. Calculated on a dry weight basis, the potassium contents of the perfused and immersed muscles do not differ markedly from their contents *in vivo*. There is, however, a very definite increase in the sodium content of the perfused muscle, but this is no more than expected as a result of the increased mannitol space.

Maclagan (1962) found that with cat tenuissimus muscle *in vivo*, infusion of depolarizing drugs produced a steady level of neuromuscular block, but no measurements of drug plasma levels were taken. In contrast, the same muscle *in vitro* exhibited a biphasic neuromuscular block similar to that described by Jenden (1955), but at no time was the level of block steady despite the application of a constant concentration of drug. Maclagan (1962) suggested that the difference between responses *in vivo* and *in vitro* might be due to deterioration. Later Gibberd (1966) suggested that a steady level of block with decamethonium was characteristic of administration via the capillaries both *in vivo* and *in vitro*. However, in this study it was not found possible to produce a steady block in the perfused diaphragm *in vitro*. This is in agreement with the finding of Creese & Maclagan (1970) who did not find a steady level of block in rat muscle *in vivo* despite constant plasma levels of decamethonium. It would therefore appear that a true steady level of block cannot be achieved in the rat by a constant concentration of decamethonium in contrast to the neuromuscular block produced by competitive blocking drugs such as tubocurarine (Holmes, Jenden & Taylor, 1951).

A neuromuscular block in which the muscle wholly or partially recovers despite the continued application of the drug has been demonstrated both in rat muscle *in vivo* (Creese & Maclagan, 1970) and in the immersed muscle *in vitro* (Gibberd, 1966). It is therefore surprising that in this study and previously (Gibberd, 1966), recovery from the block has not been demonstrated in the perfused rat diaphragm *in vitro*. The difference is probably not due to deterioration since the potassium content of the perfused and immersed preparations do not differ significantly. It has been suggested that the recovery phenomenon is due to repolarization of the muscle membrane (Gissen & Nastuk, 1966). Repolarization of the end-plate region in rat diaphragm despite a constant concentration of decamethonium has been shown by Thesleff (1955). It is of interest to speculate that repolarization is dependent upon the active transport of sodium which is stimulated by high concentrations of potassium (Horowicz & Gerber, 1965; Garrahan & Glynn, 1967). Following the depolarizing action of decamethonium the efflux of potassium is increased and the external potassium concentration is raised (Paton, 1956). It may be that, due to the kinetics of potassium washout (see below), an abnormally high external potassium concentration exists for some time in the immediate vicinity of the sodium 'pump' in the immersed preparation but not in the perfused. Hence local variations in external potassium concentrations might explain the difference in the type of responses to decamethonium. However, there is some doubt as to whether decamethonium produces a block by prolonged post synaptic depolarization in the rat (Derkx, Bonta & Lagendijk, 1971; Humphrey, 1973). It may be that in the rat depolarizing agents produce neuromuscular block by a pre-synaptic action on motor nerve terminals (Galindo, 1971).

It is well known that isolated mammalian muscle does not under normal conditions, give a twitch response to acetylcholine when added to the fluid bathing the external surface of the muscle. However, retrograde injection of acetylcholine in the isolated, normally innervated, rat diaphragm will provide a twitch (Paterson, 1965) similar to that produced *in vivo* by "close-arterial" injection (Brown, Dale & Feldberg, 1936; Brown, 1937). The difference in response to acetylcholine when applied via the capillaries may be associated with the rate of change of concentration at the motor end-plate (Paterson, 1965). This implies that the concentration rises more rapidly in the first few seconds with the perfused preparation where the diffusion path is shorter. However, the present study has been concerned with the entry of drug at the end-plate region and for this process the time uptake curves for both preparations are similar and the rates of uptake do not differ significantly.

This is in accord with the conclusion that the permeability of the end-plate region to decamethonium is similar to that of sodium (Creese & England, 1970). It is known that muscle cells are considerably more permeable to potassium than sodium and in rat muscle the estimated sodium/potasium permeability ratio is 0.006 (Creese, El-Shafie & Vrbová, 1968). The high permeability to potassium ions results in a marked discrepancy between the overall rate of exchange of ^{42}K in a whole muscle and the rate at which the isotope would exchange if all the fibres had free access to the medium (Keynes, 1954; Creese, Hashish & Scholes, 1958). The fact that exchange rates for potassium ions may be diminished by intracellular diffusion was demonstrated by Creese (1960). In rat diaphragms, Creese (1960) was able to show marked differences in the ^{42}K content of the superficial and deep layers after loading the muscle for various times with ^{42}K. Similarly, Klaus, Lullman & Muscholl (1960) demonstrated that the rate constant of the ^{42}K loss was increased up to 4-fold by retrograde perfusion of the rat diaphragm. Hence, the finding that the rate of uptake of decamethonium is *not* increased in the perfused preparation indicates that measurements made on immersed muscles do not need corrections for lags produced by intracellular diffusion. The present results therefore suggest that decamethonium behaves not like potassium, which has a high permeability, but like sodium in which the permeability is low and extracellular diffusion relatively fast, so that the uptake is usually not affected by diffusion lags. This is further supported by the autoradiographic finding that decamethonium uptake is uniform throughout the whole muscle after immersion in labelled decamethonium for 60 minutes (Humphrey, 1972).

The significance of the entry of decamethonium which has been shown *in vivo* (Creese & Maclagen, 1970) and *in vitro* (Humphrey, 1972) is still not clear. Cookson & Paton (1968) have suggested that entry occurs as a result of a non-specific change in permeability at the end-plate region caused by the agonist. Against this concept is the work carried out by Mackay & Taylor (1970) with labelled C_2 and C_6 (homologues of decamethonium with two and six methylene groups respectively). Both these compounds have been shown to have no significant uptake in mouse muscle even in the presence of pharmacological concentrations of decamethonium. It would therefore appear that decamethonium does not increase the permeability of the end-plate region to all ions. A second fact which suggests that entry is not the result of a non-specific permeability change is the finding that the ethyl analogue of decamethonium, decaethonium, is taken up almost as rapidly as decamethonium and yet has almost no effect on membrane potential (MacKay & Taylor, 1970). The kinetics of decamethonium entry show carrier-like properties (Creese & England, 1970), but the relationship between entry and pharmacological response remains to be determined.

I am very grateful to Mrs Kate Welch for technical assistance and to the Wellcome Trust and the Medical Research Council who, through grants to Professor R. Creese, supported this research. This work was included in a thesis submitted to the University of London for the degree of Doctor of Philosophy.

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(Received November 8, 1974)

Br. J. Pharmac. (1975), 54, 375-381

THE EFFECTS OF CYCLIC *N*-2-*O*-DIBUTYRYL-ADENOSINE 3′,5′-MONOPHOSPHATE, ADRENALINE AND AMINOPHYLLINE ON THE ISOMETRIC CONTRACTILITY OF THE ISOLATED HEMIDIAPHRAGM OF THE RAT

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1 *N*-2-*O*-dibutyryl adenosine 3′,5′-monophosphate (db cyclic AMP), adrenaline and aminophylline produce a potentiation of the tension developed (T_d) and the maximum rate of rise of tension (dT/dt *max*) in the rat isolated diaphragm during indirect electrical stimulation. Aminophylline and db cyclic AMP also produce the same effect during direct stimulation.

2 Propranolol produced a depression of the action of adrenaline on T_d and dT/dt *max* during indirect stimulation of the diaphragm. On the other hand, the potentiating actions of db cyclic AMP and of aminophylline on T_d and dT/dt *max* during indirect stimulation were unaffected by propranolol.

3 The results support the idea that cyclic AMP may be involved not only in regulating the processes associated with synthesis, mobilization and storage of transmitter in the motor nerve terminal, but also in modifying some metabolic processes which regulate the function of the contractile elements.

Introduction

It has been shown previously that cyclic *N*-2-*O*-dibutyryl-adenosine 3′,5′-monophosphate (db cyclic AMP) potentiates the response of the rat isolated diaphragm preparation to indirect stimulation (Varagić & Žugić, 1972). Parallel to this, db cyclic AMP has also been found to produce a significant decrease in the concentration of glycogen in the diaphragm (Varagić, Žugić & Mršulja, 1972). Cyclic adenosine-monophosphate (cyclic AMP) itself did not affect either the response of the diaphragm to phrenic nerve stimulation, or the concentration of glycogen in the diaphragm.

The dibutyryl derivative of cyclic AMP has also been found to increase the amplitude of the endplate potential in the isolated diaphragm. It has therefore been suggested that cyclic AMP might play a role in the process of acetylcholine release and also in the well known 'defatiguing effect' of adrenaline (Goldberg & Singer, 1969).

The interaction of catecholamines and methylxanthines in the cyclic AMP system has been described by Sutherland and his collaborators (Robison, Butcher & Sutherland, 1971).

In the present experiments attention was paid to recording of more precise parameters of the isometric muscle contractility tension development (T_d) and maximum rate of rise of tension (dT/dt *max*). The effects of three substances, adrenaline, aminophylline and cyclic AMP, known to affect the adenyl cyclase-cyclic AMP system, were recorded and correlated.

Methods

The isolated phrenic nerve-diaphragm preparation was set up as described by Bülbring (1946). The diaphragm preparation was suspended in a 10 ml bath at 36° C, in Tyrode solution with a double amount of glucose and bubbled with pure oxygen. The composition of the Tyrode solution was as follows (mM): NaCl 136.7, KCl 2.81, $CaCl_2$ 1.8, $MgCl_2$ 0.105, NaH_2PO_4 0 417, $NaHCO_3$ 11.9, glucose 11.101.

The diaphragm was stimulated indirectly with pulses of 0.8 ms duration and a frequency of 0.1 to 0.15 Hz. The isometric contractions were recorded with a Statham Universal Transducing Cell and displayed on a Physiograph IV polygraph paper. Both tension development (T_d) and maximum rate of rise of tension (dT/dt *max*) were recorded simultaneously. The differential of tension with respect to time was recorded by

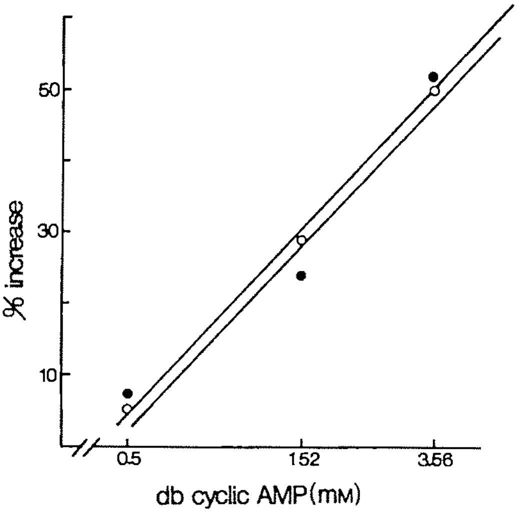

Figure 1 The effects of dibutyryl (db) cyclic AMP on tension development (T_d) (●) and the maximum rate of rise of tension (dT/dt *max*) (○) in the isolated hemidiaphragm of the rat during indirect stimulation. Abscissae: concentrations of db cyclic AMP in the bath in mM (log scale). Ordinates: percentage increase in the parameters measured.

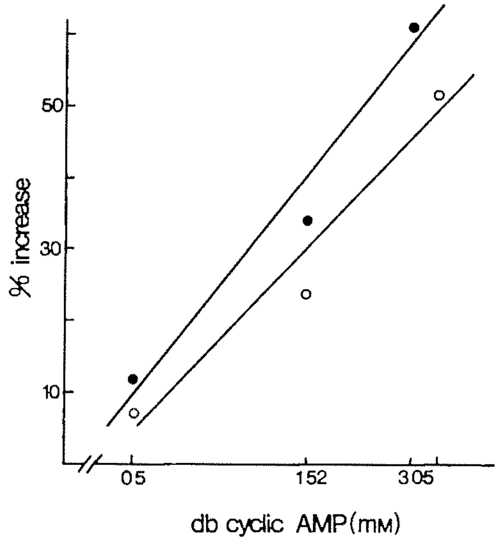

Figure 2 The effects of dibutyryl (db) cyclic AMP and propranolol on T_d of the isolated hemidiaphragm of the rat during indirect stimulation. Abscissae: concentrations of db cyclic AMP in the bath in mM (log scale). Ordinates: percentage increase in T_d. (○) effect of db cyclic AMP; (●) effect of db cyclic AMP in the presence of propranolol (1 μg/ml).

means of a Differentiator Coupler Type 7301 (Narco-Bio-Systems, Inc.), which provides the true mathematical derivative of analog signal voltages.

Direct stimulation with pulses of 1-1.5 ms duration, 0.1 to 0.15 Hz, was applied by means of two palador wires; the diaphragm was secured to one wire and the other was in the bathing solution. In these experiments (+)-tubocurarine was added to the bath in a concentration sufficient to block neuromuscular transmission completely (3 μg/ml, 4 x 10^{-6}M).

The following substances were used: cyclic *N*-2-*O*-dibutyryl adenosine 3′,5′-monophosphate-monosodium salt (Boehringer), cyclic adenosine 3′,5′-monophosphate, aminophylline, adrenaline hydrochloride, (+)-tubocurarine chloride, adenosine 5′-triphosphate sodium salt (Koch-Light Laboratories), nicotinic acid and propranolol hydrochloride.

Results

The effests of db cyclic AMP on indirect stimulation

Dibutyryl cyclic AMP was added to the bath in concentrations ranging from 0.5 to 3.56 mM in nine experiments. These concentrations of db cyclic AMP produced simultaneously an equal and parallel dose-dependent increase both in tension development (T_d) and in the maximum rate of rise of tension (dT/dt *max*) during indirect stimulation. When the percentage increase of these two parameters was plotted against the molar concentration of db cyclic AMP on a log scale, two close parallel lines were obtained (Figure 1).

The maximum increase in T_d and dT/dt *max* was reached 10 to 20 min after addition of db cyclic AMP to the bath and lasted for about 40 minutes. The percentage increase of these two parameters of contractility varied from preparation to preparation even when the same standard concentration of db cyclic AMP was present in the bath.

Cyclic AMP itself (not the dibutyryl derivative) in concentrations from 0.5 to 3 mM did not affect either T_d or dT/dt *max*.

Adenosine 5′-triphosphate (ATP) (0.5-3 mM) produced in three experiments a short lasting increase both in T_d and dT/dt *max*, followed by a depression. The increase in these two parameters lasted only a few minutes. It was evident that the effects of ATP and of db cyclic AMP differed both qualitatively and quantitatively.

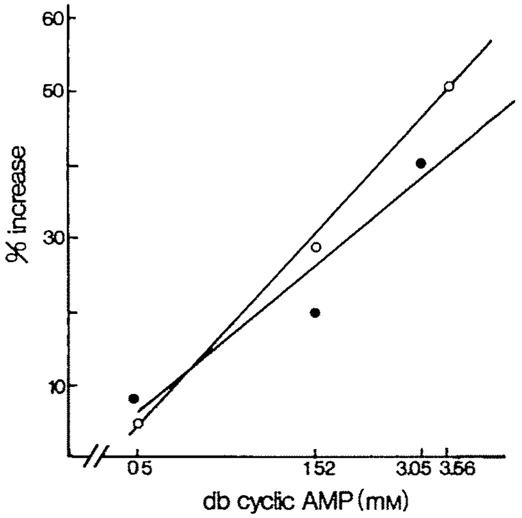

Figure 3 The effect of dibutyryl (db) cyclic AMP and propranolol on $\mathrm{d}T/\mathrm{d}t$ *max* of the isolated hemidiaphragm of the rat during indirect stimulation. Abscissae: concentrations of db cyclic AMP in the bath in mM (log scale). Ordinates: percentage increase in $\mathrm{d}T/\mathrm{d}t$ *max*. (○) effect of db cyclic AMP; (●) effect of db cyclic AMP in the presence of propranolol (1 µg/ml).

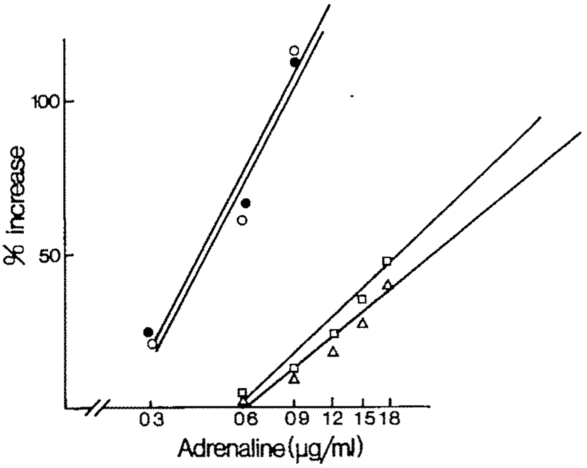

Figure 4 The effects of adrenaline and propranolol on T_d and $\mathrm{d}T/\mathrm{d}t$ *max* of the isolated hemidiaphragm of the rat during indirect stimulation. Abscissae, concentration of adrenaline, in µg/ml (log scale). Ordinates: percentage increase in the parameters measured. (●) effects of adrenaline on T_d and (○) $\mathrm{d}T/\mathrm{d}t$ *max*, (□) effect of adrenaline on T_d in the presence of propranolol (1 µg/ml); (△) effect of adrenaline on $\mathrm{d}T/\mathrm{d}t$ *max* in the presence of propranolol (1 µg/ml).

Sodium butyrate was added to the bath in amounts corresponding to the amount of butyrate in the db cyclic AMP molecule (three experiments). These concentrations of butyrate (0.1-0.4 mg/ml) did not affect either T_d or $\mathrm{d}T/\mathrm{d}t$ *max*.

Dibutyryl cyclic AMP and propranolol

To investigate the site of action of db cyclic AMP, propranolol was used in order to block the β-adrenoceptors. In a concentration of 1 µg/ml propranolol itself did not affect the response of the diaphragm to indirect stimulation.

This concentration of propranolol did not significantly alter either the effect of db cyclic AMP on T_d or its action on $\mathrm{d}T/\mathrm{d}t$ *max*. The results of four experiments are presented in Figures 2 and 3. In all these experiments propranolol was added to the bath 5-7 min before the addition of db cyclic AMP.

The effect of adrenaline

Adrenaline has long been known to produce facilitation during indirect stimulation of the diaphragm (Bülbring, 1946). In the present experiments adrenaline (0.3 to 0.9 µg/ml) produced an equal and parallel increase both in T_d and $\mathrm{d}T/\mathrm{d}t$ *max*. If the percentage increase of these two parameters was plotted against the concentration of adrenaline on a log scale, two parallel straight lines were obtained (Figure 4).

In the presence of propranolol (1 µg/ml) the responses to adrenaline, both in T_d and $\mathrm{d}T/\mathrm{d}t$ *max*, were significantly smaller, the log dose lines being shifted to the right (Figure 4). This type of response indicates the competitive type of antagonism between adrenaline and propranolol. The results shown in Figure 4 were obtained from five experiments.

The effect of aminophylline

In 15 experiments aminophylline, in concentrations ranging from 250 to 1000 µg/ml, was found to increase both tension development and the maximum rate of rise of tension during indirect stimulation of the diaphragm. The increase in both parameters was equal and parallel. When the percentage increase in T_d and $\mathrm{d}T/\mathrm{d}t$ *max* was plotted against the concentration of aminophylline, two parallel straight lines were obtained (Figure 5).

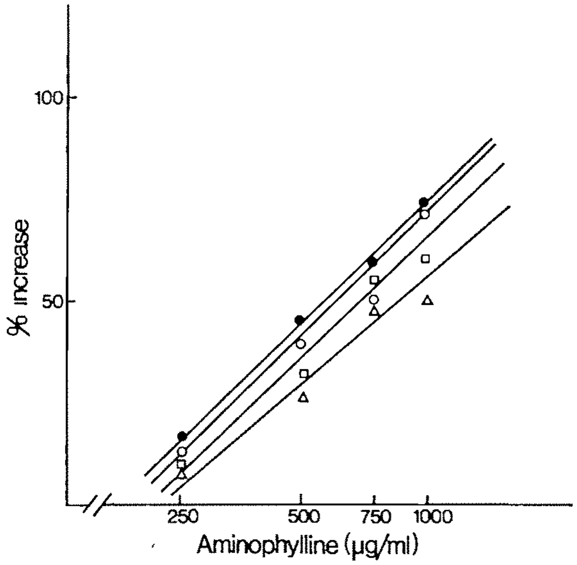

Figure 5 The effects of aminophylline and propranolol on T_d and dT/dt *max* of the isolated hemidiaphragm of the rat during indirect stimulation. Abscissae. concentrations of aminophylline in μg/ml (log scale) Ordinates. percentage increase in the parameters measured. (●) effects of aminophylline on T_d and (○) dT/dt *max*, (□) effect of aminophylline on T_d in the presence of propranolol (1 μg/ml); (△) effect of aminophylline on dT/dt *max* in the presence of propranolol (1 μg/ml).

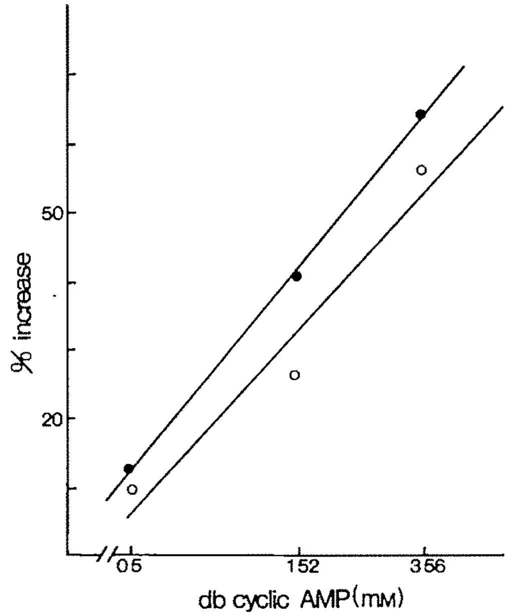

Figure 6 The effect of increasing concentrations of dibutyryl (db) cyclic AMP (log scale) on the percentage increase in T_d (●) and dT/dt *max* (○) of the isolated hemidiaphragm of the rat during direct stimulation.

Similar effects were obtained in the presence of propranolol (1 μg/ml). This concentration of propranolol produced no change in the response to aminophylline, the values for T_d and dT/dt *max* were similar to those obtained with aminophylline alone with no significant shift of the dose-response curves (Figure 5).

Dibutyryl cyclic AMP and direct stimulation

In 9 of a series of 12 hemidiaphragms, taken from six animals, db cyclic AMP (0.5 to 3.56 mM) produced an increase both of T_d and dT/dt *max* during direct stimulation. The results from these nine experiments are shown in Figure 6. It can be seen that parallel lines are obtained if the percentage increase is plotted against concentrations of db cyclic AMP on a log scale. In the other 3 of the 12 hemidiaphragms the same concentrations of db cyclic AMP produced no change in either T_d or dT/dt *max*. It should be noted that in these three preparations even aminophylline (0.5 mg/ml) produced only an insignificant increase in T_d and dT/dt *max*.

Nicotinic acid (0.5 to 2 mg/ml) was found to depress or completely block the effect of db cyclic AMP on the isometric contractility of the iolated hemidiaphragm (four experiments).

Aminophylline and direct stimulation

In seven experiments aminophylline (250 to 1000 μg/ml) was tested during direct stimulation. These concentrations of aminophylline regularly produced a parallel increase both in T_d and dT/dt *max*.

Discussion

The three substances studied in the present experiments, db cyclic AMP, adrenaline and aminophylline, all potentiated the tension developed (T_d) and the maximum rate of rise of tension (dT/dt *max*) of the rat isolated diaphragm when stimulated indirectly. Dibutyryl cyclic AMP and aminophylline also produced this effect during direct stimulation. In the original concept of the cyclic AMP system, as proposed by Robison *et al.* (1971), all three substances are intimately interconnected. Catecholamines have been shown to stimulate adenyl cyclase, whereas xanthine derivatives including aminophylline inhibit the enzyme phosphodiesterase and thus produce an accumulation of cyclic AMP (Butcher & Sutherland, 1962). Adrenaline and aminophylline supposedly act through the intracellular cyclic AMP produced. It might therefore be expected that the general intracellular mediator, i.e. cyclic AMP, would produce the same type of response as the substances which produce either its increased synthesis (adrenaline) or decreased breakdown (aminophylline).

Although cyclic AMP alone did not produce any change in response of the isolated diaphragm to indirect stimulation, this is to be expected because nucleotides and other anionic phosphorylated compounds penetrate cell membranes poorly, if at all (Robison *et al.*, 1971). This generalization may not apply equally to all nucleotides and to all cells (Robison, Butcher, Oye, Morgan & Sutherland, 1965). Dibutyryl cyclic AMP contains lipid-soluble fatty acid residues which facilitate its passage across cellular membranes and at the same time increase its resistance to enzymatic hydrolysis by phosphodiesterase (Posternak, Sutherland & Henion, 1962; Falbriard, Posternak & Sutherland, 1967). This may explain why db cyclic AMP potentiated T_d and dT/dt *max* in the present experiments, whereas cyclic AMP itself did not.

It was also shown that propranolol, a β-adrenoceptor blocking agent, affected the action of adrenaline on T_d and dT/dt *max*, causing a significant shift of the log dose-response lines to the right. This possibly indicates a competitive type of antagonism between adrenaline and propranolol on β-adrenoceptors, present in the diaphragm somewhere on the excitation-contraction pathway. It has already been suggested that adenyl cyclase itself might serve a β-adrenoceptor function (Robison, Butcher & Sutherland, 1967). On the other hand, propranolol did not affect either the effect of db cyclic AMP or the response to aminophylline of the diaphragm when stimulated indirectly. Both db cyclic AMP and aminophylline produced practically the same effects on T_d and dT/dt *max*, no matter whether propranolol was present or not. This is hardly surprising since the processes affected by aminophylline (Haugaard & Hees, 1966) and by cyclic AMP (Robison *et al.*, 1971) are distal to the adenyl cyclase system.

Breckenridge, Burn & Matshinsky (1967) were the first to suggest that cyclic AMP within the motor nerve ending might augment the release of acetylcholine at the terminal membrane. Evidence supporting this hypothesis is the finding that addition of cyclic AMP or db cyclic AMP to the bathing medium of an isolated nerve muscle preparation increases transmitter release (Goldberg & Singer, 1969). The frequency of miniature end plate potentials is increased in response to db cyclic AMP and theophylline (Goldberg & Singer, 1969). It has also been shown that adrenaline increases the frequency and amplitude of the miniature endplate potentials (Krnjević & Miledi, 1958). Increased quantities of acetylcholine are released in response to adrenaline at the neuromuscular and ganglionic junctions (Krnjević & Miledi, 1958, Birks & MacIntosh, 1961). Therefore our results in which db cyclic AMP, adrenaline and aminophylline were found to increase T_d and dT/dt *max* during indirect stimulation could be explained in terms of an increased release of transmitter at the neuromuscular junction in the isolated diaphragm. This conclusion is strongly supported by the finding of Wilson (1974) that db cyclic AMP causes a significant increase in the quantum content of the first endplate potential in the mobilization rate and in the releasable store of transmitter.

On the other hand, it has been reported that adenosine reduces transmitter release (Ginsborg & Hirst, 1972). Adenosine also increases the amount of cyclic AMP in brain tissue (Sattin & Rall, 1970) and probably a similar effect might be expected at the nerve terminal. All these data are taken to indicate that actions of db cyclic AMP, adrenaline and aminophylline on the transmitting process are not the sole factors contributing to the potentiation of T_d and dT/dt *max* in the diaphragm during indirect stimulation. We have shown that db cyclic AMP and aminophylline also produce an increase in these two parameters even after direct stimulation. Cyclic AMP is known to have a role in the processes of glycogenolysis and lipolysis (Sutherland & Rall, 1960; Butcher, Ho, Meng & Sutherland, 1965; Reynolds & Haugaard, 1967). In the presence of ATP, cyclic AMP converts phosphorylase b to active phosphorylase a, the levels of which are increased by adrenaline (Hornbrook & Brody, 1963). It is therefore possible that the potentiating effects of db cyclic AMP and aminophylline on T_d and dT/dt *max*, particularly during direct stimulation of the diaphragm, might be due, at least partly, to activation of metabolic processes which provide the energy for contraction.

Besides inhibiting phosphodiesterase, the methylxanthines can induce calcium release from intracellular stores (Bianchi, 1961; Isaacson & Sandow, 1967) The transmitter release is known to be calcium-dependent (Hubbard, Jones &

Landau, 1968), but calcium also activates the contractile mechanism. In cardiac tissue db cyclic AMP, as well as adrenaline, has been shown to produce a significant increase in ^{45}Ca uptake (Meinertz, Nawrath & Scholz, 1973). These actions mean that more calcium is available both for transmitter release and to the contractile elements. Thus the potentiation of T_d and dT/dt *max* by aminophylline and by db cyclic AMP, particularly during direct stimulation, might be due to calcium-dependent changes in the muscle action potential, with increase in calcium release during the spike and depression of the mechanical threshold for activation of the contractile elements. Such an effect has already been shown for caffeine (Sandow, 1970).

Our experiments therefore indicate that cyclic AMP may be involved not only in regulating the processes associated with synthesis, mobilization and storage of transmitter in the nerve terminal, but also in modifying some metabolic process (or processes), which regulates the function of the contractile elements.

V.M.V. wishes to thank the Wellcome Trust for providing a grant which partially covered the cost of the equipment. Thanks are also due to Miss Ljubica Nikolić for skilful technical assistance. This work was financed by ZMNU of SR Serbia.

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(Received October 14, 1974)

Br. J. Pharmac. (1975), 54, 383-388

SOME CHARACTERISTICS OF PRE- AND POST-SYNAPTIC INHIBITORY RECEPTORS AT THE HERMIT CRAB NEUROMUSCULAR JUNCTION

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1 The effects of γ-aminobutyric acid (GABA), β-guanidinopropionic acid (βGP) and picrotoxin on the pre- and post-synaptic receptors of the hermit crab neuromuscular junction were studied quantitatively using electrophysiological techniques. Reductions in excitatory junction potential (e.j.p.) amplitude and membrane resistance were measured simultaneously from the same cells.

2 The pre- and post-synaptic receptors were activated by the same order of concentration of GABA, whereas βGP stimulated the pre-synaptic receptors at a concentration ten times lower than was required to affect the post-synaptic membrane.

3 Picrotoxin appeared to antagonize the pre-synaptic action of βGP in a competitive manner. The affinity constants (± s.e. mean) for picrotoxin 5×10^{-6}M and 2×10^{-4}M were 6.80 (± 0.46) $\times 10^5$ M^{-1} and 6.42 (± 1.8) $\times 10^5$ M^{-1} respectively.

4 The effect of GABA on e.j.p. amplitude also appeared to be antagonized competitively by picrotoxin whereas the post-synaptic effect was antagonized in a non-competitive manner.

5 Possible differences in the nature of the pre- and post-synaptic receptors are discussed.

Introduction

In a previous publication we demonstrated that both pre- and post-synaptic mechanisms of inhibition exist at the hermit crab neuromuscular junction (Earl & Large, 1974). Furthermore, we considered that neural inhibition was achieved largely by the pre-synaptic mechanism, whereas the effect of exogenous γ-aminobutyric acid (GABA), the inhibitory transmitter at the crustacean neuromuscular junction, could be accounted for to a great extent by its ability to reduce the post-synaptic membrane resistance.

The pre- and post-synaptic inhibitory sites have certain features in common; they are both blocked by picrotoxin (Takeuchi & Takeuchi, 1969; Earl & Large, 1974) and in both cases the action of GABA brings about a selective increase in chloride conductance (Boistel & Fatt, 1958; Takeuchi & Takeuchi, 1966). However, there appears to be at least one difference; at the crayfish neuromuscular junction, the GABA analogue β-guanidinopropionic acid (βGP) attenuates excitatory junction potentials (e.j.ps) without affecting the post-synaptic membrane resistance (Dudel, 1965) thus indicating a purely pre-synaptic inhibitory action. Furthermore, βGP antagonizes the action of GABA at the post-synaptic site (Feltz, 1971). Therefore, βGP appears to be a GABA-like agonist pre-synaptically but a competitive antagonist post-synaptically. Presumably there is a difference between either the pre- and post-synaptic receptors or the receptor-ionophore links.

In this study we carried out experiments to make a quantitative assessment of the sensitivity of pre- and post-junctional receptors to GABA-mimetics and picrotoxin

Methods

Measurement of e.j.ps and membrane resistance

The abductor muscle of the large claw of the hermit crab (*Eupagurus bernhardus*) was used to study e.j.ps and membrane resistance. Details of the techniques are described elsewhere (Earl & Large, 1974). Briefly, e.j.ps were evoked by stimulation of the thin nerve bundle via a suction electrode and were recorded in the conventional manner with an intracellular microelectrode filled with 3M KCl. For measurement of membrane resistance, this electrode was used to record the voltage displacements produced by passing hyperpolarizing current pulses through a second intracellular KCl electrode. Input resistance was read as the slope of the current-voltage plot obtained when both electrodes were impaled in the middle of the fibre, less than 100 μm apart. Membrane resistance was calculated by the simplified method of Takeuchi & Takeuchi (1967). After successive applications of Ringer solution containing increasing concentrations of GABA or βGP, dose-response curves of percentage reduction of membrane resistance or e.j.p. amplitude against log concentration of GABA or βGP were plotted One minute after the addition of each concentration of agonist, readings were taken of both current-voltage relationship and e.j.p. amplitude from the same fibre. The latter was estimated by recording several trains of e.j.ps, to allow time for complete facilitation, and then averaging the amplitude of the last three e.j.ps within a train. In those experiments in which the blocking action of picrotoxin was investigated, the dose-response curves were plotted after the preparation had been exposed to picrotoxin for 2 minutes. The composition of the Ringer solution was (mM): NaCl 445, KCl 12.2, $CaCl_2$ 29.6, $MgCl_2$ 5.75, $NaHCO_3$ 1.79.

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Calculation of the contribution of pre- and post-synaptic components to e.j.p. depression

Activation of post-synaptic inhibitory receptors by agonists will trigger a fall in the resistance of the post-synaptic membrane; if the contribution of this post-synaptic effect to the reduction of the e.j.p. amplitude can be estimated quantitatively, then pre-junctional inhibition can be detected from the excess depression of the e.j.p. The membrane current associated with an e.j.p. is partly a displacement current that charges the membrane capacity and partly an ionic current. In order to calculate the maximum possible contribution of the post-synaptic component of inhibition we have assumed that all the synaptic current passes through the membrane resistance. Using this analysis, a drug-induced reduction in e.j.p. amplitude which cannot be accounted for by a decrease in post-synaptic membrane resistance must be due to stimulation of the pre-synaptic inhibitory receptors. In fact the analysis will over-estimate the contribution of post-synaptic inhibition in reducing the amplitude of e.j.p. as will be discussed later.

If the membrane capacitance is ignored the equivalent electrical circuit of the post-synaptic membrane of the crab neuromuscular junction is given in Figure 1. The resting membrane potential of hermit crab fibres is usually around −70 mV and the membrane resistance $\simeq 10^4\,\Omega$. The equilibrium potential for the excitatory transmitter, in the crayfish, is $\sim$+ 15 mV (Takeuchi & Onodera, 1973) while that of the inhibitory transmitter is $\sim$ −70 mV (Dudel & Kuffler, 1961). In channel A, R_T represents the synaptic resistance produced by the action of the excitatory transmitter. Closing switch 1 represents the action of the excitatory transmitter alone and sets up a current

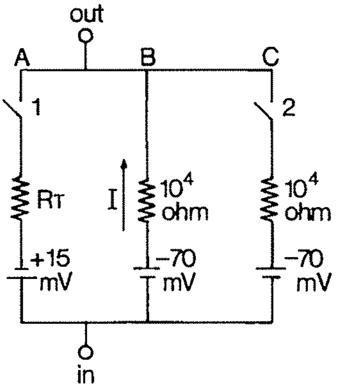

Figure 1 Equivalent electrical circuit of the post-synaptic membrane of the crustacean neuromuscular junction. Channel B represents the elements of the non-junctional membrane; A & C represent the pathways opened by the actions of the excitatory and inhibitory transmitters respectively.

$$I = \frac{15-(-70)}{R_T + 10^4}\ \text{mA}$$

this would lead to a depolarization of

$$\frac{85}{R_T + 10^4} \cdot 10^4\ \text{mV}$$

Since the amplitude of the unfacilitated e.j.p. is of the order of 1 mV, this means that R_T would have a value of 84.10^4 ohms, i e. $R_T \gg R_m$, the resting membrane resistance. Let us now consider the effect of closing switches 1 and 2 together. By the use of simultaneous equations, the calculated depolarization (e.j.p.) produced by the excitatory transmitter is reduced from 1 mV to approximately 0.5 mV. The shunt resistance of 10^4 ohms, represented in the inhibitory channel C, (produced by the appropriate concentration of GABA) corresponds to an effective reduction of the membrane resistance by 50%, and therefore it can be concluded that the e.j.p. amplitude is directly proportional to the membrane resistance. When the % reduction in e.j.p. amplitude is greater than the concomitant depression of post-synaptic

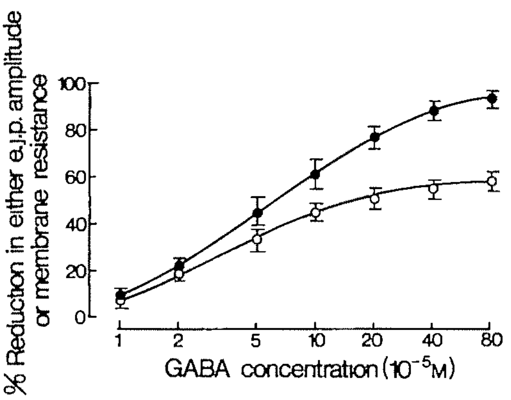

Figure 2 Effect of γ-aminobutyric acid (GABA) on e.j.p. amplitude and membrane resistance. The ordinate scale is the % reduction in either e.j.p. amplitude (●) or membrane resistance (○) and the abscissa scale is the GABA concentration plotted on a log scale. Each point represents the mean of 12 determinations. Vertical lines show s.e. mean.

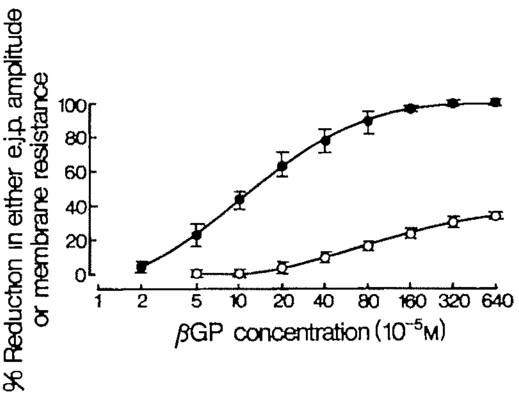

Figure 3 Effect of β-guanidinoproprionic acid (βGP) on e.j.p. amplitude and membrane resistance. The ordinate scale is the % reduction in either e.j.p. amplitude (●) or membrane resistance (○) and the abscissa scale is the βGP concentration plotted on a log scale. Each point represents the mean of 7 determinations. Vertical lines show s.e. mean.

membrane resistance, the difference must be due to a pre-synaptic effect

Results

Effects of GABA and βGP on e.j.p. amplitude and post-synaptic membrane resistance

The threshold concentration of GABA for depression of both e.j.p. amplitude and membrane resistance was always 1-2 x 10^{-5}M (Figure 2). Whereas it was usually possible to attenuate the e.j.ps by 90%, the fall in membrane resistance was only 50-60%. These GABA-induced effects were not always totally reversed on washing, the maximal recovery taking 5-30 minutes. In those experiments where measurement of more than one dose-response curve was intended, e.j.p. depression was not allowed to proceed by more than 70%; in these instances, it was usually possible to attain the control e.j.p. amplitude and membrane resistance values on washing.

From the curves in Figure 2, it can be seen that for small doses of GABA (10^{-5} to 10^{-4}M), the reduction in post-synaptic membrane resistance can account for the major part of the e.j.p. attenuation, but for higher doses the pre-synaptic inhibitory component becomes increasingly more significant However, the pre- and post-synaptic receptors appear to be activated by the same order of concentration of GABA, as in most experiments there was a small pre-synaptic inhibitory component (about 10% of the total inhibition) with small doses of GABA.

In contrast to the results with GABA, the threshold concentration of βGP (Figure 3) to reduce the post-synaptic membrane resistance was higher than that to attenuate the e.j.ps (in the order of 10-fold) Therefore, it appears that there is a difference between the pre- and post-synaptic inhibitory receptors in the hermit crab neuromuscular junction as in the crayfish. The inhibitory action of βGP is of an entirely pre-synaptic origin at concentrations below 20-40 x 10^{-5}M and even at higher concentrations, the post-synaptic effect accounts for only a small proportion of the inhibition.

Effect of picrotoxin on the inhibition mediated by GABA and βGP

The ability of picrotoxin to antagonize the pre- and post-synaptic inhibitory mechanisms was investigated. The two concentrations of picrotoxin used (5 x 10^{-6} and 2 x 10^{-4}M) were chosen because in earlier studies (Earl & Large, 1974) we had shown that picrotoxin 5 x 10^{-6}M produced a relatively weak antagonism of the post-synaptic action of GABA and it was predicted that 2 x 10^{-4}M would cause a marked block

The first series of experiments was carried out with βGP since its action (at low concentrations) can be considered a purely pre-synaptic phenomenon Figure 4 shows that picrotoxin produced a dose-dependent antagonism of βGP;

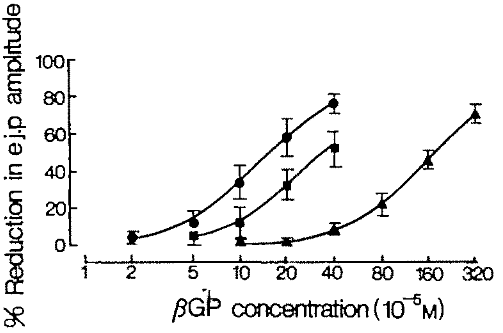

Figure 4 Effect of picrotoxin on the inhibitory action of β-guanidinoproprionic acid (βGP). The ordinate scale is the % reduction in e.j.p. amplitude and the abscissa scale is the βGP concentration plotted on a log scale Each point represents the mean of 6 determinations. Vertical lines show s.e. mean. (●) Control; (■) 5×10^{-6}M picrotoxin; (▲) 2×10^{-4}M picrotoxin.

there was a parallel shift to the right of the curves with increasing concentrations. It was possible to achieve maximal reduction in e.j.p. amplitude, even in the presence of 2×10^{-4}M picrotoxin, on the occasions when larger doses of βGP were added, thus suggesting that the antagonism is of a competitive nature. From the results of the individual experiments obtained with βGP it was possible to estimate the affinity constant, K_A, of picrotoxin for the pre-synaptic receptors. The values of K_A were calculated using the equation $r_A^n - 1 = X_B^m \cdot K_A$ (Rang, 1971) where r_A is the dose-ratio, n is the number of molecules of agonist that combine with the receptor, X_B is the molar concentration of antagonist and m is the number of molecules of antagonist that combine with the receptor. Takeuchi & Takeuchi (1969) suggested that two molecules of GABA or one molecule of picrotoxin combine with the post-synaptic receptor, therefore we let $n = 2$ and $m = 1$ for our calculations. The calculated affinity constants for picrotoxin 5×10^{-6}M and 2×10^{-4}M with βGP as agonist were 6.80 (± 0.46) x 10^5M^{-1} and 6.42 (± 1 8) x 10^5M^{-1} respectively (mean ± s.e. of 6 determinations). These values were not significantly different at the 95% probability level when compared by Student's *t*-test. If other values of n, e g. 1, 3 or 4 were used, there was less agreement between the K_A values of the high and low picrotoxin concentrations, which would be consistent with values for n and m used earlier.

When the effect of GABA on e.j.ps was challenged with the same concentrations of picrotoxin (Figure 5a), there was also a parallel shift in the curves. Also, as with βGP, it was possible to achieve the maximal effect on e.j.p. attenuation in the presence of picrotoxin. Further, if the affinity constants for picrotoxin 5×10^{-6}M and 2×10^{-4}M against GABA depression of e.j.ps are calculated from the curves in Figure 5a, one obtains the values 6.8 x 10^5M^{-1} and 5.0 x 10^5M^{-1} respectively. These are very similar to the values calculated for βGP which acts purely on the pre-synaptic receptors.

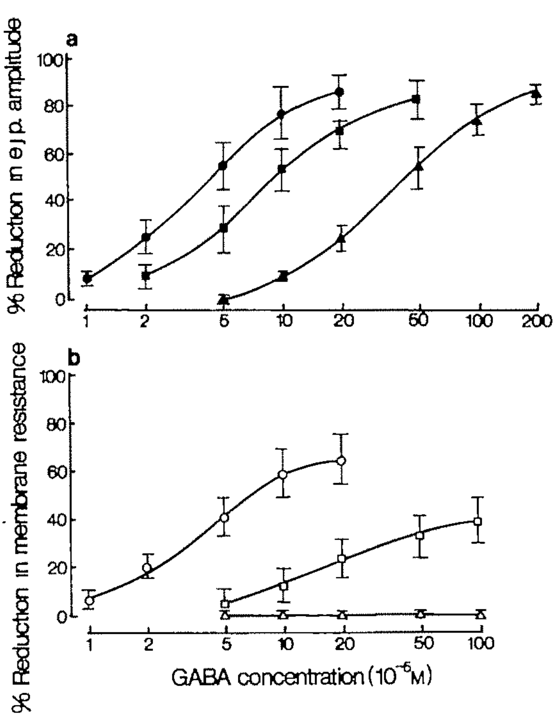

Figure 5 Effect of picrotoxin on the inhibitory action of γ-aminobutyric acid (GABA). The ordinate scales represent the % reduction in either e.j p. amplitude (a) or membrane resistance (b) and the abscissa scale is the GABA concentration plotted on a log scale. Each point represents the mean of 6 determinations Vertical lines show s.e. mean. (●) and (○) Control; (■) and (□) 5×10^{-6}M picrotoxin, (▲) and (△) 2×10^{-4}M picrotoxin.

This is in direct contrast to the effect of GABA on the post-synaptic membrane resistance (Figure 5b). In these experiments (with picrotoxin 5×10^{-6}M), there was the characteristic non-parallel shift with a reduced maximum previously observed with picrotoxin (Takeuchi & Takeuchi, 1969, Earl & Large, 1974). In fact, in the presence of picrotoxin 2×10^{-4}M, there was total block of the post-synaptic GABA effect even though the e.j.ps had been reduced by 80-90%. It is of interest that picrotoxin 5×10^{-6}M causes a larger shift of the curve for the post-synaptic GABA effect (Figure 5b) than it does with βGP a pre-synaptic agonist (Figure 4). As this antagonism of GABA by picrotoxin at the post-junctional receptor appears to be non-competitive, the equation used earlier for calculation of the affinity constant does not hold.

A further point is that there was a direct effect of the higher concentration of picrotoxin on e.j.p. amplitude, the amplitude was increased by a mean value of 35% (range 10-60% in 12 experiments), with little accompanying change in the post-synaptic membrane resistance. The most probable explanation for this effect is that there is a large tonic discharge of GABA from the pre-synaptic inhibitory terminals and little onto the post-synaptic receptors.

Discussion

With low concentrations of GABA, although the major part of the e.j.p. reduction can be accounted for by a post-synaptic action, there was still a small pre-synaptic component suggesting that both populations of receptors were similar in their sensitivity to GABA. At the crayfish neuromuscular junction, βGP competitively antagonizes the action of GABA suggesting that the two compounds combine with the same receptor (Dudel, 1965; Feltz, 1971). Therefore, our finding that βGP stimulates the pre-synaptic receptor in lower concentrations than are required to activate the post-synaptic sites suggests that there is a difference between the two populations of receptors. It should be noted that, in the hermit crab, βGP produces a post-synaptic membrane conductance increase and therefore differs from the crayfish; presumably this is due to a species difference.

Furthermore, there appears to be a difference in the pre- and post-synaptic receptors with respect to their antagonism by picrotoxin. The results with βGP suggest that picrotoxin antagonizes the pre-synaptic inhibitory actions in a competitive way, while the post-synaptic effect of GABA is blocked in a non-competitive manner. There are two possible explanations for this. Firstly, as suggested above, the pre- and post-synaptic receptors are in some way chemically different, and/or, secondly, there are many 'spare receptors' pre-synaptically and few or none post-synaptically.

In the presence of picrotoxin the shift of the GABA post-synaptic membrane resistance curve was greater than that of either the GABA or βGP e.j.p. curves, suggesting that picrotoxin has a greater affinity for the post-synaptic than the pre-synaptic receptors It is interesting that the qualitative and quantitative characteristics of the antagonism of GABA-induced e.j p. attenuation by picrotoxin are similar to those of βGP (purely pre-synaptic). This would suggest that the inhibitory action of GABA is also largely pre-junctional, despite the accompanying large fall in post-synaptic membrane resistance. This can be accounted for if the rising phase of the e j.p. is due largely to a capacitative current, with little current passing through the membrane resistance, in fact an equation can be derived where the e j.p. amplitude is independent of the membrane resistance (see equation, (5a) in Ginsborg, 1973).

The observation that one can achieve total attenuation of e.j.p. amplitude in the presence of high concentrations of picrotoxin indicates the potency of the pre-synaptic inhibitory mechanism. Moreover, this offers a better explanation than limited access of picrotoxin, for the difficulty in blocking pre-synaptic rather than post-synaptic inhibition previously reported (Earl & Large, 1974).

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RAPID EFFECTS OF ENVIRONMENTAL DISTURBANCE ON RAT PLASMA UNESTERIFIED FATTY ACID AND TRYPTOPHAN CONCENTRATIONS AND THEIR PREVENTION BY ANTILIPOLYTIC DRUGS

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1 Changes of plasma unesterified fatty acid (UFA) and tryptophan concentration in group-housed rats following removal of their cage-mates and the effects of antilipolytic drugs on these changes were investigated.

2 Removal of group-housed 24 h fasted rats but not fed rats from cages resulted in increased plasma UFA concentration in the remaining rats which was associated with significant increases of the proportion of free tryptophan but significant falls of total tryptophan concentration. These rapid changes were not associated with brain tryptophan changes. Plasma tyrosine concentration was unaffected.

3 The fall of plasma tryptophan did not appear to be due to passage into red cells as erythrocyte tryptophan concentration remained unchanged.

4 Plasma UFA concentrations correlated positively and significantly with corticosterone concentrations which were also increased following removal of cage-mates.

5 Plasma UFA increases and tryptophan changes in the fasting rats were both prevented by nicotinic acid or propranolol. Corticosterone concentration was increased by nicotinic acid but unaffected by propranolol.

6 The possible importance of these rapid changes of plasma tryptophan and of their prevention by antilipolytic drugs is discussed.

Introduction

Tryptophan is normally bound largely to albumin in the plasma of man (McMenamy, Lund & Oncley, 1957) and other warm blooded vertebrates (Fuller & Roush, 1973). Only the unbound form is directly available for transport to tissues. When plasma unesterified fatty acid (UFA) concentration increases, the free fraction of tryptophan also increases as UFA binds to albumin which results in weakened binding of tryptophan (McMenamy & Oncley, 1958; Curzon, Friedel & Knott, 1973).

Plasma UFA derives from lipolysis of body fat, a mechanism initiated by sympathetic activity and mediated by cyclic adenosine-3′,5′-monophosphate (AMP). This process has been described as a 'unique confluence of biochemistry with psychology' (Brodie & Maickel, 1963). Its importance is perhaps emphasized by the demonstration that it can alter the availability of tryptophan to the brain and thus lead to altered synthesis therein of the neurotransmitter 5-hydroxytryptamine (5-HT). Various drugs can alter brain 5-HT metabolism apparently because they alter the binding of plasma tryptophan to albumin by interacting with the sympathetic – cyclic AMP system. Also, in stress situations increased plasma UFA may be associated with increase of brain tryptophan and 5-HT turnover, e.g. in the rat when deprived of food or immobilized (Knott & Curzon, 1972; Curzon & Knott, 1974). These are prolonged or extreme changes in the physical state of the animal. We now find that the mere removal of rats from a cage can cause rapid and considerable increases of UFA and alteration of the binding and amount of tryptophan in the plasma of their remaining cage-mates. This paper describes a study of these changes and of their prevention by antilipolytic drugs.

Methods

Male Sprague-Dawley rats, 160-180 g (Carworth Europe, Alconbury, Huntington), caged in groups of either three of four, were kept in an acoustically lagged housing at 22°C ± 2 and fed a diet of Oxoid 41B *ad libitum*. At least four days after their arrival in the laboratory when body weight had risen to 180-200 g, food but not water was removed from some of the cages at 16 h 00 min and both fed and food-deprived groups were killed by guillotine the next day, between 16 h 00 min-17 h 00 min, in sequence at 2 min intervals, cage by cage. Animals were usually killed in the room containing their housing but out of sight of the remaining rats. Rats which had been singly housed on their arrival in the laboratory were also deprived of food for 24 h and killed at 2 min intervals. In additional groups of fasted rats, nicotinic acid (50 mg/kg as a suspension in 0.9% w/v NaCl solution (saline)) or propranolol (1 mg/kg) were injected intraperitoneally 1 h before the animals were killed. Control rats were injected with saline (2.5 ml/kg).

Brains were removed and plasma samples prepared as previously described (Curzon, Joseph & Knott, 1972).

Analytical methods

Tryptophan was determined by the method of Denkla & Dewey (1967) in whole plasma and also in plasma ultrafiltrates prepared by centrifuging 1.0 ml plasma in a CF50 Diaflo membrane cone (Amicon) at 800 *g* for 30 min at room temperature Tryptophan was determined in packed red cells by a modification of the approach of Denkla & Dewey. In preliminary experiments it was found that initial protein precipitation and extraction as used in the plasma tryptophan method led to very low recoveries from red cells. A modified method was therefore developed (Kantamaneni, unpublished work) Approximately 1 g of packed red cells was weighed, homogenized with 1 ml deionized water using an X-1020 homogenizer (International Laboratory Apparatus Gmb, Göttingen, Germany) and 20 ml acid butanol added. After agitation of the suspension with a vortex mixer and centrifugation, 5 ml of the supernatant was back-extracted into 2.0 ml 0.1 N HCl after addition of 10 ml heptane To 0.2 ml of the acid phase, 2.0 ml 10% w/v trichloroacetic acid was added. After centrifugation for 5 min, 0.1 ml 1.8% v/v formaldehyde and 0.1 ml 10% w/v trichloroacetic acid containing 3 mM $FeCl_3$ was added to 1.0 ml supernatant. The mixture was heated in a boiling water bath and the tryptophan fluorophor measured as in the method of Denkla & Dewey (1967). Heating was begun immediately after adding the $FeCl_3$.

Plasma UFA was determined by the method of Laurell & Tibbling (1967). Brain tryptophan and tyrosine and plasma tyrosine, were measured as previously described (Curzon *et al.*, 1972). Plasma corticosterone was determined by the method of Mattingly (1962) scaled down for use with 0.25 ml plasma samples.

Results

Effect of removal of cage-mates on rat plasma unesterified fatty acid and on tryptophan disposition

Fed grouped rats. Plasma UFA, total tryptophan and free tryptophan concentrations did not show significant relationships with order of removal from cages (Figure 1). However the combination of non-significant upward and downward trends in plasma free and total tryptophan respectively led to a small but significant increase of % free tryptophan with order of removal. Brain tryptophan was unaffected by order of removal.

Fasted grouped rats Plasma UFA progressively and significantly increased with order of removal and total tryptophan progressively and significantly fell so that its mean concentration in the last rats in each cage was about two-thirds of that found for the initially removed rats killed 6 min earlier. Free tryptophan showed an upward trend though this was not significant while % free tryptophan rose significantly. Brain tryptophan was unaffected by order of removal. Mean values for plasma UFA, free tryptophan, % free tryptophan and brain tryptophan of 1st, 2nd, 3rd and 4th killed fasted rats were greater than those for corresponding groups of fed animals though not all of these differences were significant (see legend to Figure 1). Although plasma UFA concentration of 1st killed fasted rats was not significantly different from that of 1st killed fed rats, standard deviations were large and numbers of determinations small. However, when 1st killed fasted rats were compared with 1st, 2nd, 3rd and 4th killed fed rats combined, then significant differences were found (fasted, 0.662 ± 0.090 mEq/l, $n = 8$, fed, 0.495 ± 0.140 mEq/l, $n = 14$.

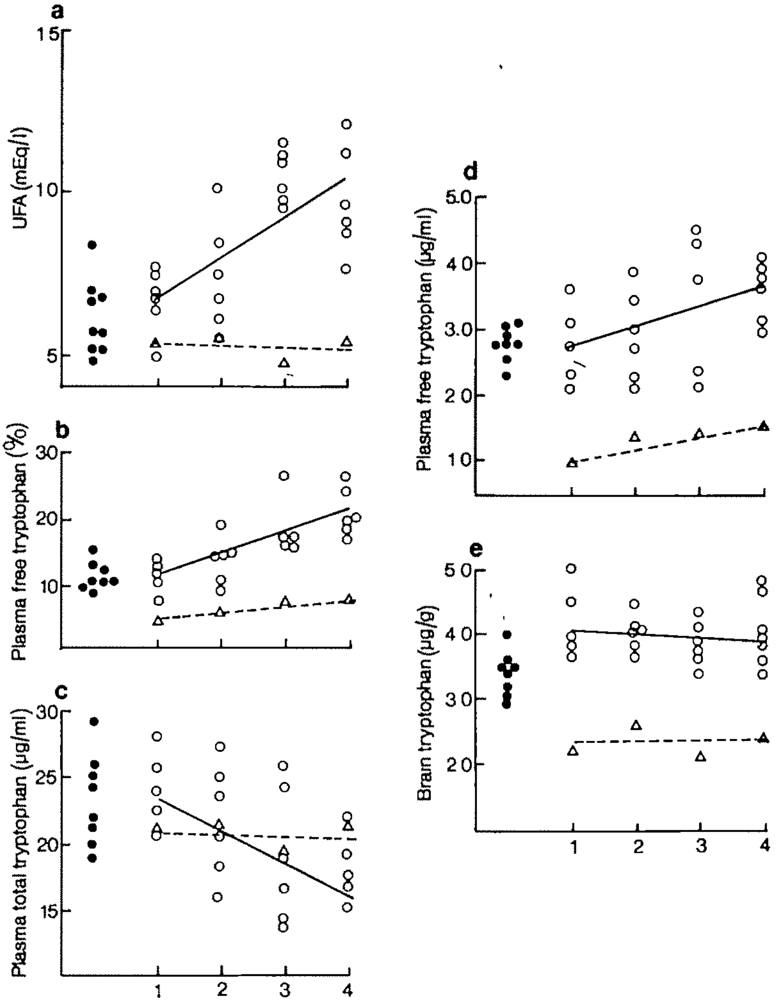

Figure 1 Effect of order of removal of rats from cage on plasma unesterified fatty acid (UFA) concentrations and plasma and brain tryptophan concentrations. *X* axis shows order of killing. Animals were killed at 2 min intervals. (○) Fasted rats housed 4/cage, individual values are shown; regression lines for these points are unbroken. (△) Fed rats housed 4/cage, each point is the mean of three observations; the best fit line is broken. Standard deviations are not shown to improve clarity. (●) Fasted singly housed rats, each point shows an individual value.

Correlations with order of killing: (a) Plasma UFA. fasted rats, $r = 0.6744$, $n = 24$, $P < 0.001$, fed rats, $r = 0.0271$, $n = 12$, NS. (b) Plasma % free tryptophan: fasted rats, $r = 0.7309$, $n = 22$, $P < 0.001$; fed rats, $r = 0.6083$, $n = 12$, $P < 0.05$. (c) Plasma total tryptophan. fasted rats, $r = -0.5612$, $n = 23$, $P < 0.01$, fed rats, $r = -0.1411$, $n = 12$, NS. (d) Plasma free tryptophan: fasted rats, $r = 0.4195$, $n = 22$, NS; fed rats, $r = 0.5540$, $n = 12$, NS. (e) Brain tryptophan: fasted rats, $r = -0.1054$, $n = 24$, NS; fed rats, $r = -0.0251$, $n = 12$, NS. Values for fasted and fed rats compared by Student's t test are given below.

Order of removal	*Total tryptophan*	*Free tryptophan*	*% Free tryptophan*	*UFA*	*Brain tryptophan*
1st	NS	<0.01	<0.001	NS	<0.001
2nd	NS	<0.02	<0.02	NS	<0.001
3rd	NS	<0.05	<0.001	<0.001	<0.001
4th	NS	<0.001	<0.001	<0.001	<0.02

$P < 0.02$). As plasma UFA of fed rats showed no sign of a relationship with order of killing, this statistical treatment seems reasonable.

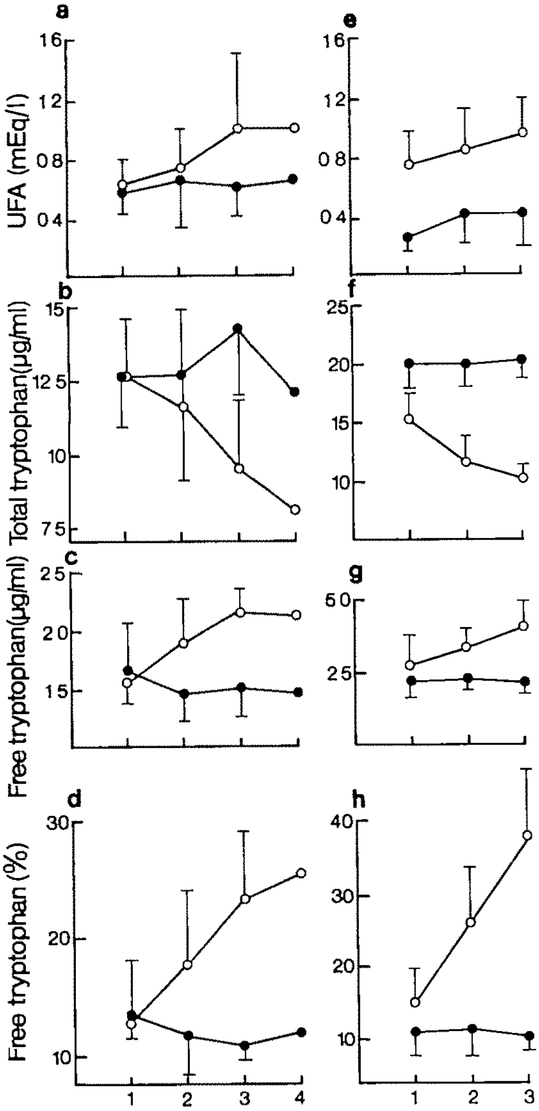

Figure 2 Effect of antilipolytic drugs on the changes of plasma unesterfied fatty acid (UFA) and trytophan concentrations following removal of cage-mates of fasted rats. *X* axis shows order of killing. Animals were killed at 2 min intervals. (a)-(d) Effects of propranolol, (○) saline; (●) propranolol. Rats were housed either 3 or 4/cage. Each point is the mean of 6 observations except the last point which represents the mean of 2 animals only. Vertical lines represent one s.d. Propranolol (1 mg/kg) was given intraperitoneally and rats were killed 1 h after injection. (e)-(h) Effects of nicotinic acid, (○) saline; (●) nicotinic acid. Rats were housed 3/cage. Each point is the mean of 6 observations. Nicotinic acid (50 mg/kg) was given intraperitoneally and rats were killed 1 h after injection. Vertical lines represent one s.d.

Fasted singly housed rats. Mean values for all of the above parameters were comparable with those found for the first killed members of fasted groups of rats. Singly housed fed rats were not studied in this experiment but in a separate experiment it was found that singly housed fasted rats had significantly ($P < 0.001$) higher brain tryptophan concentration (4.97 ± 0.33 μg/g wet wt., $n = 5$) than did singly housed fed rats (3.59 ± 0.27 μg/g wet wt., $n = 6$). (Results ± one s.d.)

Modifications of experimental procedure. The possibility that the plasma changes in fasted grouped rats were artefactual or related to trivial aspects of the experimental situation were investigated with animals housed in different enclosures or removed from cages by one investigator and killed by another investigator in a different room. In addition, chemical determinations were made in random order. Results of all experiments were consistent with those shown in Figure 1.

Erythrocyte tryptophan. It was thought possible that the fall of plasma tryptophan concentration of fasted rats on removal of cage-mates was simply due to movement of newly freed tryptophan into red cells either *in vivo* or during the separation of plasma. However red cell tryptophan did not rise (Table 1).

Prevention by antilipolytic drugs of changes of plasma unesterified fatty acid and tryptophan following removal of cage-mates of fasted rats

Propranolol. Injection of the β-adrenoceptor blocking agent, propranolol (1 mg/kg i.p.), 1 h before killing prevented the changes of both plasma UFA and tryptophan following removal of cage-mates of fasted rats (Figure 2). Plasma UFA and tryptophan concentrations of initially removed rats were unaffected by propranolol and brain tryptophan concentration was similar to that of fasted rats injected with saline (Table 2).

Nicotinic acid. Injection of nicotinic acid (50 mg/kg i.p.) 1 h before killing similarly prevented or opposed the effects of removal of cage-mates of fasted rats (Figure 2). In agreement with previous experiments (Curzon & Knott, 1974), the plasma UFA concentration of initially removed nicotonic acid-treated rats was significantly lower and total tryptophan concentration significantly higher than values for saline-treated fasted controls. Plasma UFA concentrations of subsequently removed nicotinic acid-treated rats were not significantly higher than values for initially removed rats while plasma tryptophan concentrations remained similar to those of the initially removed animals and as before (Curzon & Knott, 1974) brain tryptophan was significantly lower than in saline-treated fasted control rats (Table 2).

Other effects of fasting, removal of cage-mates and antilipolytic drugs

Plasma tyrosine. To determine whether the plasma tryptophan changes of fasted rats on previous removal of cage-mates were specific to this amino acid, plasma tyrosine determinations were also made on animals caged in groups of three, killed at 2 min intervals (Table 3). Values were not significantly related to order of removal. In addition, injection of propranolol (1 mg/kg i.p.) 1 h before killing was without effect on plasma tyrosine concentration although nicotinic acid (50 mg/kg) caused moderate but significant increases. Percentage tyrosine increases were comparable with those of tryptophan (Figure 2).

Plasma corticosterone. Removal of cage-mates of fasted rats was associated with increased plasma corticosterone concentration in the remaining rats (Table 4). Plasma corticosterone concentrations were positively and significantly correlated with both order of removal and plasma UFA concentra-

Table 1 Effect of removal of cage-mates on plasma and red cell tryptophan of 24 h fasted rats

Order of removal	*Plasma total tryptophan (μg/ml)*	*Plasma free tryptophan (μg/ml)*	*Red blood cell tryptophan (μg/g)*
1st	13.04 ± 2.39 (8)	1.82 ± 0.27 (8)	6.22 ± 0.50 (8)
2nd	10.81 ± 2.18 (8)	2.48 ± 0.68 (8)	6.64 ± 2.41 (8)
3rd	10.21 ± 1.18 (8)*	2.75 ± 0.35 (8)	6.84 ± 0.97 (8)

Animals were caged in groups of 3. Results are expressed as means ± one s.d. Numbers of determinations shown in parentheses. Results compared by Student's t test, *, significantly less than values for first removed rats $P < 0.02$.

Table 2 Effects of propanolol and nicotinic acid on brain tryptophan of 24 h fasted rats

Injected	*Brain tryptophan (μg/g)*
Saline (12)	3.68 ± 0.37
Propranolol, 1 mg/kg (12)	3.59 ± 0.45
Saline (18)	4.23 ± 0.88
Nicotinic acid, 50 mg/kg (18)	3.33 ± 0.58*

Injections were made i.p. and rats killed 1 h later. Volumes injected were 2.5 ml/kg body wt. Animals were caged in groups of 3 and results on 1st, 2nd and 3rd removed rats combined. Results are expressed as means ± one s.d. Numbers of determinations shown in parentheses. Results compared by Student's t test. * $P = 0.001$.

Table 3 Effects of removal of cage-mates and of propranolol and nicotinic acid on plasma tyrosine of fasted rats

	Order of removal		
	1st	*2nd*	*3rd*
Injected		*Plasma tyrosine (μg/ml)*	
Saline	13.92 ± 1.60 (8)	14.94 ± 3.51 (4)	15.72 ± 3.73 (8)
Propranolol, 1 mg/kg	13.60 ± 1.48 (4)	12.80 ± 1.11 (4)	14.10 ± 1.90 (3)
Nicotinic acid, 50 mg/kg	17.74 ± 3.05 (4)*	20.83 ± 3.09 (4)*	20.02 ± 1.99 (4)

General conditions as in Table 2.
* $P < 0.05$.

tion. Treatments with propranolol and nicotonic acid 1 h before killing had markedly different effects on corticosterone concentrations. Thus, while propranolol-treated rats had concentrations not significantly different from those of saline-treated fasted control animals, nicotinic acid caused a large increase of plasma corticosterone.

Discussion

The previous finding that fasting for 24 h leads to increased brain trytophan (Curzon, Joseph & Knott, 1972; Knott & Curzon, 1972) has been confirmed in both singly and group-housed rats. Results also show that in fasted rats lipolytic mechanisms are readily enhanced by environmental disturbance so that the mere removal of rats from cages is followed by large and rapid increases of plasma UFA in their former cage-mates. Increased plasma UFA within minutes of exposure to stress situations occurs in human subjects (Court, Dunlop & Leonard, 1971; Taggart & Carruthers, 1971; Nimmo, Kirby & Lassers, 1973) while rats have been previously reported to show plasma UFA changes following minimal environmental disturbance (Barrett, 1964).

Two factors are probably responsible for the lack of increase of plasma UFA on removal of cage-mates of fed rats. Firstly, the starved animals were much more obviously responsive to disturbance and therefore sympathetic activity may have increased much more in these animals than in fed rats on removal of cage-mates. Secondly, similar increases of sympathetic activity have greater lipolytic effect in starved than in fed rats (Brodie, Krishna & Hynie, 1969).

In circumstances such as fasting (Knott & Curzon, 1972) and experimental hepatic coma (Curzon, Kantamaneni, Winch, Rojas-Bueno, Murray-Lyon & Williams, 1973) or following the administration of many drugs (Curzon & Knott, 1974; Knott & Curzon, 1975), the weakened binding of plasma tryptophan to albumin which results from an increase of plasma UFA appears to lead to increased brain tryptophan. Furthermore, a group of uninjected rats showed significant positive correlations between free trytophan in plasma and trytophan concentration in brain regions (Curzon, Kantamaneni, Fernando, Woods & Cavanagh, 1975). Therefore it seemed possible that the rapid plasma UFA increase on removal of cage-mates or on other brief disturbances could lead to similar transient brain tryptophan changes. Present findings do not indicate this. The rapid increases of plasma UFA, though they led to liberation of tryptophan from albumin binding as shown by large increases of percentage free tryptophan, were also associated with a decrease of plasma total tryptophan so that absolute concentrations of plasma free tryptophan only increased relatively slightly and brain tryptophan did not increase within the brief period over which measurements were made. The fall of plasma total tryptophan presumably resulted from a shift of the newly freed tryptophan into intracellular compartments other than the brain. Thus rapid transient changes of plasma UFA on brief stress, unlike the more prolonged changes in previoulsy studied circumstances, do not appear to cause comparable brain tryptophan changes.

Table 4 Effects of removal of cage-mates and of propranolol and nicotinic acid on plasma corticosterone of fasted rats

		Order of removal			
		1st	*2nd*	*3rd*	*1st, 2nd, 3rd combined*
	Injected	*Plasma corticosterone (µg/100 ml)*			
Expt 1.	–	32.7 ± 12.7 (8)	36.3 ± 10.2 (8)	46.1 ± 12.1 (8)	
Expt 2.	Saline	12.1 ± 5.0 (4)	22.4 ± 7.7 (3)	20.6 ± 9.0 (4)	18.0 ± 8.1 (11)
	Propranolol, 1 mg/kg	14.3 ± 8.3 (4)	19.7 ± 15.7 (4)	10.8 ± 7.1 (3)	15.3 ± 10.9 (11)
Expt 3.	Saline	22.3 ± 4.3 (4)	16.8 ± 9.5 (4)	29.6 ± 10.4 (4)	22.8 ± 9.4 (12)
	Nicotinic acid, 50 mg/kg	42.2 ± 12.7 (4)	50.0 ± 4.7 (4)	50.5 ± 18.6 (4)	47.3 ± 13.1 (12)*

General conditions as in Table 2

Expt 1. Relationship between corticosterone concentration and order of removal. $r = 0.4460$, $n = 24$, $P < 0.05$. Relationship between corticosterone and UFA concentrations (latter values not shown) $r = 0.5583$, $n = 24$, $P < 0.01$.

* $P < 0.001$.

Another plasma amino acid, tyrosine, did not show similar changes to those of tryptophan. This finding is consistent with the fall of plasma tryptophan being related to its liberation from albumin, as tryptophan is the only amino acid present in plasma in both protein-bound and free states (McMenamy *et al.*, 1957). Also the prevention of the rapid plasma tryptophan changes by the antilipolytic drugs propranolol and nicotinic acid is consistent with the changes being caused by an increase of plasma UFA.

Although the β-adrenoceptor blocking agent, propranolol, prevented the increase of plasma UFA on removal of cage-mates, it did not decrease plasma UFA in initially removed rats; nicotinic acid decreased UFA even in these rats. This observation indicates that sympathetic changes were responsible for the rapid UFA increase on removal of cage-mates but not for the underlying increase of plasma UFA on fasting and agrees with the results of Brodie *et al.* (1969) who showed that the increase of UFA caused by fasting is unaffected by β-receptor blockade but is opposed by nicotinic acid which acts directly on fat cells by reducing the level of cyclic AMP.

Unlike propranolol, nicotinic acid decreased brain tryptophan concentration, in agreement with earlier work on fasted rats (Curzon & Knott, 1974; Fernando, Joseph & Curzon, 1975). The roles of the decrease of plasma UFA and the increase of corticosterone in these tryptophan changes require further investigation.

These results demonstrate how readily the disposition of tryptophan is influenced by a brief and largely psychological disturbance. They also suggest that propranolol and nicotinic acid and its derivatives may be useful tools in research on tryptophan metabolism. For example, they could be used to prevent changes mediated by the peripheral sympathetic-UFA mechanism and thus facilitate study of purely centrally provoked changes of brain trytophan and 5-HT metabolism in stress situations. The stabilizing effect of these drugs on plasma tryptophan levels and the influence of nicotinic acid on the availability of tryptophan to the tissues (Curzon & Knott, 1974) may be of interest in relation to the reported alteration of trytophan metabolism in schizophrenic crises (Brune & Himwich, 1962) and the beneficial effect of propranolol in schizophrenia (Yorkston, Zaki, Malik, Morrison & Havard, 1974). Although there have been claims that nicotinamide is beneficial in schizophrenia (Hoffer, 1962) they have not been confirmed (e.g. Ban, 1973).

Finally, the results described here indicate that often disregarded environmental disturbances may be relevant to the design and interpretation of experiments on tryptophan and 5-HT metabolism.

We thank the MRC for financial support and Mr P.H. Hutson for skilled technical assistance.

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(Received March 12, 1975)

Br. J. Pharmac. (1975), 54, 397-399

COMPARATIVE BIOASSAY OF PROSTAGLANDIN E_2 AND ITS THREE PULMONARY METABOLITES

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The relative potencies of prostaglandin E_2 and its metabolites 15-keto PGE_2, 13,14-dihydro-15-keto-PGE_2 and 13,14,dihydro-PGE_2 were investigated on isolated smooth muscle preparations. These were rat stomach strip, colon and uterus, chick rectum, guinea-pig ileum, trachea and pulmonary artery and rabbit aorta and pulmonary artery. 15-keto PGE_2 was equiactive or 1-1.8 times more potent than prostaglandin E_2 in relaxing guinea-pig trachea but otherwise the three metabolites were less active than prostaglandin E_2 on all preparations.

Introduction Prostaglandins of the E series are metabolized by guinea-pig lung homogenates to yield two types of metabolites, these are 15-keto-13,14-dihydro- and 13,14-dihydro-derivatives (Ånggård, 1971). The metabolic sequence involves the initial formation of a 15-keto-prostaglandin E metabolite Although this metabolite is not seen when prostaglandin E_2 is completely metabolized by guinea-pig lung homogenates, it is the sole product of metabolism by swine lung homogenates.

Metabolism of prostaglandin in guinea-pig lung results in its biological inactivation (Piper, Vane & Wyllie, 1970) and since this inactivation is extremely efficient (90-95%) it has generally been assumed that the pulmonary metabolites of prostaglandins have little biological activity It has been shown that 13,14-dihydro-15-keto-PGE_1 has little spasmogenic activity on many tissues. However, 13,14-dihydro-PGE_1 shows appreciable biological activity, especially when tested on guinea-pig, rat and rabbit blood pressure (Ånggård, 1966; Pike, Kupiecki & Weeks, 1967). Dawson, Lewis, McMahon & Sweatman (1974) have reported that the 15-keto metabolite of prostaglandin $F_{2\alpha}$ is a potent bronchoconstrictor agent and appears to possess greater biological activity than the parent on several isolated smooth muscle preparations. We have therefore examined the effects of the three pulmonary metabolites of prostaglandin E_2 (15-keto-PGE_2, 13,14-dihydro-15-keto-PGE_2 and 13,14-dihydro-PGE_2) on a variety of isolated smooth muscle preparations.

Methods The tissues commonly used for detection and bioassay of prostaglandins, rat stomach strip, chick rectum and rat colon, were arranged for superfusion in banks of three. Their order was varied from experiment to experiment. The tissues were superfused at 5 ml/min with Krebs solution which was warmed to 37°C and gassed with a mixture of 5% CO_2 and 95% O_2. A combination of antagonists to 5-hydroxytryptamine, catecholamines, acetylcholine and histamine were infused into the Krebs solution superfusing the tissues to make them more specific for prostaglandins (Piper & Vane, 1969). Prostaglandin E_2 and its metabolites were given as random 2 min infusions into the superfusing Krebs solution. A dose cycle of 10-15 min was adopted. Contractions of the assay tissues were measured auxotonically by Harvard smooth muscle transducers and displayed on a Watanabe 6-channel pen recorder. The sensitivity of the recorder was adjusted to a suitable level for the first experiment and then kept unaltered for the remaining experiments. The metabolites were also tested on several other smooth muscle strips superfused by the method described. These were guinea-pig ileum, trachea and pulmonary artery, and spirally cut strips of rabbit aorta and pulmonary artery. Female rats were brought into oestrus by subcutaneous injection of stilboestrol 1 mg/kg 18 h before use. Uteri were divided into two horns and superfused at 5 ml/min with de Jalon's solution.

The purity of prostaglandin E_2 and its metabolites was checked by thin layer chromatography in the AI and AIII systems of Grêen & Samuelsson (1964).

Results All three metabolites were active on the rat stomach strip, chick rectum and rat colon (Figure 1). The 13,14-dihydro metabolite had roughly 0.2, 15-keto-prostaglandin E_2 0.1, and 13,14-dihydro-15-keto-PGE_2 about 0.01 times the activity of the parent substance on all three tissues No differential responses of the tissues were seen. The reactivity of the tissues was unchanged by their position in the cascade.

The metabolites were inactive on rabbit aorta (2 experiments) and rabbit and guinea-pig pulmonary arteries (1 experiment each) in doses up to 1 μg/ml. Prostaglandin E_2 was inactive in

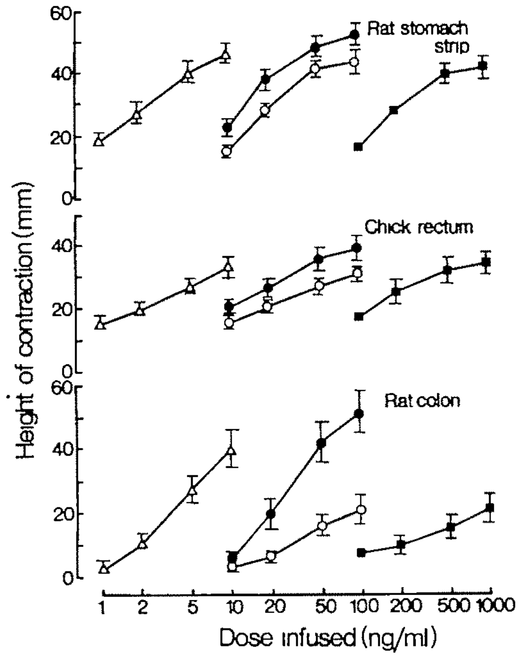

Figure 1 Comparative bioassay of prostaglandin E_2 (PGE_2) (△) and its pulmonary metabolites 13,14-dihydro-PGE_2 (●), 15-keto-PGE_2 (○) and 13,14-dihydro-15-keto-PGE_2 (■) on rat stomach strip, chick rectum and rat colon. Prostaglandin E_2 or metabolites were given as random 2 min infusions into the Krebs solution superfusing the assay tissues. The height of contraction of the assay tissues was plotted against the log dose of agonist. Each point is the mean of 8-10 experiments. Vertical bars show s.e. mean.

doses up to 100 ng/ml. 13,14-dihydro-PGE_2 was about 0.1 times as active as the parent compound on guinea-pig ileum, but the keto- and dihydro-keto-metabolites were inactive in doses up to 1 µg/ml (4 experiments). Prostaglandin E_2 contracted guinea-pig ileum with a threshold dose of 10 ng/ml.

On guinea-pig trachea, 13,14-dihydro-PGE_2 and 13,14-dihydro-15-keto-PGE_2 showed the same relative potency as on the prostaglandin assay tissues, i.e. approximately 0.1-0.2 and 0 01 times the potency of the parent respectively in relaxing this tissue. However, 15-keto-PGE_2 was 1.3 times as potent as the parent in relaxing guinea-pig trachea (4 experiments, range 0.8-1.8).

The 15-keto and 13,14-dihydro-15-keto metabolites were inactive on rat uterus in doses up to 1 µg/ml but 13,14-dihydro-PGE_2 was 0.4 times as active as prostaglandin E_2 (6 experiments, range 0.4-0.5).

Discussion The results show that two of the three pulmonary metabolites of prostaglandin E_2 have marked activity on the three assay tissues commonly used to detect parent prostaglandins. Moreover, the ratio of potency of parent and metabolites is roughly the same on all three tissues, making identification by differential assay impossible. For this reason, distinction between a relatively low level of prostaglandin E_2 and a higher level of 15-keto-PGE_2 or 13,14-dihydro-PGE_2 in biological fluids is not possible by bioassay alone. Hence, results indicating a 'release of prostaglandin' by bioassay alone without further identification should be regarded with caution. In addition the apparently high degree of inactivation of prostaglandin E_2 by isolated perfused lungs may in fact be even higher, since some of the residual activity of the effluent from the lungs may be due in part to the intrinsic activity of the metabolites formed.

Mathé & Levine (1973) have reported the release of high levels of prostaglandin metabolites from guinea-pig lung during anaphylaxis. The relatively high potency of 15-keto-PGE_2 in relaxing the guinea-pig trachea suggests that it may have a bronchodilator action when released in the lung. Since Dawson *et al.* (1974) have reported that 15-keto-$PGF_{2\alpha}$ is a more potent bronchoconstrictor agent than is prostaglandin $F_{2\alpha}$ several of the biological effects of prostaglandins released in anaphylactic shock (Piper & Vane, 1969) may be due in part to high levels of prostaglandin metabolites.

13,14-dihydro-PGE_2 has about 0.2 times the activity of prostaglandin E_2 on rat stomach strip, chick rectum and rat colon, but nearly 0.4 times the parent prostaglandin's activity on the rat uterus. Since the pulmonary metabolism of prostaglandins is increased in late pregnancy (Bedwani & Marley, 1974), relatively more of this metabolite may be formed and might contribute to the induction of labour by intravenous infusion of prostaglandin E_2.

We wish to thank Dr J.E. Pike (The Upjohn Co., Kalamazoo) for the gift of prostaglandin E_2 and its metabolites. We also thank the Medical Research Council and the Wellcome Trust for grants.

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Br. J. Pharmac. (1975), 54, 400-402

DEPOLARIZING EFFECTS OF THE IONOPHORES X-537A AND A23187 AND THEIR RELEVANCE TO SECRETION

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The ionophore X-537A depolarized frog skeletal muscle fibres about 10 mV in 10 min and a further 30 mV or so over the next 50 minutes With A23187, depolarization was relatively feeble and delayed in onset but was hastened when calcium was removed from the bathing medium. The results support conjecture that some functional responses to these ionophores are attributable, in part, to depolarization.

Introduction The ionophores X-537A and A23187, which are known to transfer calcium ions across cell membranes, elicit calcium-dependent secretion from various cells and this has generally been considered support for the view that calcium is a mediator in stimulus-secretion coupling. However, the effectiveness of the two ionophores as secretagogues does not parallel their reported abilities to transfer calcium ions. Thus, while both ionophores stimulate catecholamine output from perfused cat adrenals, X-537A is much more potent (Cochrane, Douglas, Mouri & Nakazato, 1975) even though it is the less effective calcium ionophore (Pressman, 1972, Pfeiffer, Reed & Lardy, 1974). Similarly, X-537A strongly stimulates vasopressin release from isolated neurohypophyses while A23187 does not (Nakazato & Douglas, 1974). Since depolarization is known to provide an adequate stimulus for secretion in chromaffin cells and neurohypophyseal terminals and because X-537A, unlike A23187, has the ability to transport monovalent cations, it has been suggested that the greater effectiveness of X-537A as a secretagogue in these systems may be related to an ability to depolarize (Nakazato & Douglas, 1974; Cochrane *et al.*, 1975). The purpose of the present experiments was to examine the effects of X-537A and A23187 on membrane potential. For convenience we have used frog skeletal muscle cells.

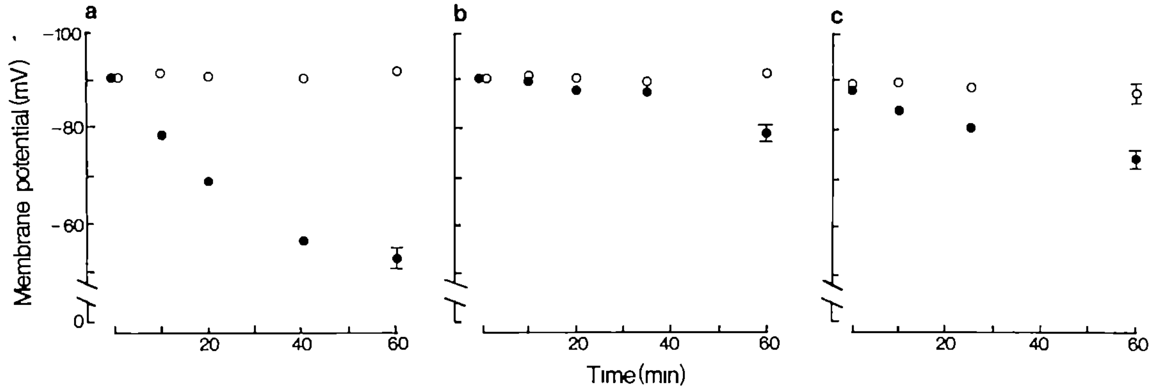

Figure 1 Depolarizing effects of the ionophores X-537A and A23187 on muscle fibres and the effect of calcium deprivation, (○) control potentials, (●) potentials in the presence of ionophore. Dimethylsulphoxide (0.2%) was present in all instances. (a) X-537A (10 μg/ml) in Ringer solution. (b) A23187 (10 μg/ml) in Ringer solution. (c) A23187 (10 μg/ml) in Ca-free Ringer solution Muscles were exposed to Ringer or Ca-free Ringer solution for 40 min before introducing the ionophore. Each symbol represents the mean potential of many fibres (10-70) from different muscles The standard error, where larger than the symbol, is indicated by vertical lines Differences between sample means were tested for significance by Student's non-paired *t*-test and where equality of sample variances did not hold a modification of this test was used according to Snedecor & Cochran (1967). Except for the values at time zero and those in (b) at 10, 20, and 35 min, all values in the presence of the ionophores are significantly different from their corresponding controls ($P < 0.01$).

Methods Membrane potentials were recorded *in vitro* from frog (*Rana pipiens*) sartorius muscle fibres bathed in Ringer or Ca-free Ringer solution at about 22°C using 3 M KCl micropipettes. The Ringer solution had the following composition (mM): NaCl, 120; KCl, 2.5; $CaCl_2$, 1.8; Tris(tris (hydroxymethyl) aminomethane), 1.0, adjusted to pH 7.3. For Ca-free Ringer solution, $CaCl_2$ was omitted. Bathing media containing X-537A and A23187 were prepared from stock solutions in dimethylsulphoxide (DMSO). The DMSO concentration never exceeded 0.2% which was without effect on membrane potential (Figure 1).

Some muscles were fixed in 10% formalin and stained paraffin sections examined by light microscopy.

Results Some 10 min after treating muscle fibres in Ringer solution with X-537A (10 μg/ml), membrane potential had fallen about 10 mV and it continued to fall progressively thereafter (Figure 1a). After 2 h, we could not record any membrane potentials greater than −20 mV. By contrast, the membrane potential of fibres bathed in Ringer solution containing A23187 (10 μg/ml) was affected little for 30 min, although by 60 min it had fallen about 10 mV (Figure 1b).

A23187 has sometimes been introduced to tissues bathed with Ca-free media to facilitate its absorption by cell membranes. We have found that calcium deprivation hastens the depolarizing effect of A23187 (Figure 1c).

Although our main concern here is with depolarization, other effects of the ionophores that we have observed deserve mention. During the first 15 min of exposure to X-537A, some fibres were seen to twitch and by 40 min many appeared swollen. After 2 h the surface of the muscle, as viewed through the dissecting microscope, was no longer smooth but had a granular appearance. Many fibres had irregular regions of contracture and some seemed ruptured. Histological examination of muscles exposed to X-537A for 2 h showed that most of the surface fibres were ruptured. Damage was less in fibres lying deeper in the muscle. With A23187 no twitching occurred. After 2 h some fibres had a beaded appearance and showed peristalsis-like movements and some seemed to be ruptured. Histological examination showed many ruptured fibres mainly at the muscle surface. The damage was less, however, than with X-537A.

Discussion The strong and abrupt depolarization produced by X-537A is probably attributable, at least in part, to this drug's ability to transport monovalent cations (Pressman, 1972; 1973) and thereby facilitate movement of sodium and potassium ions down their concentration gradients. In lipid bilayers X-537A has been shown to increase membrane conductance to monovalent and divalent cations up to 10^4 times (Célis, Estrada-O & Montal, 1974). After this manuscript had been submitted, Devore & Nastuk (1975) reported a similar depolarizing effect of X-537A on frog cutaneous pectoris muscle which they conclude is due to the inward transport of Na^+ ions. Because A23187 has little ability to transport monovalent cations, especially at physiological pH (Pfeiffer *et al.*, 1974), its relatively small and delayed depolarizing effect has probably a different explanation. One possibility is that A23187 complexes with and depletes the membrane-bound calcium needed for maintenance of normal membrane permeability and potential: that calcium deprivation hastened the depolarizing effect of A23187 supports this interpretation. A similar action may contribute to depolarization in response to X-537A. Additional effects of the ionophores that could lead to depolarization are: (1) uncoupling of oxidative phosphorylation (Reed & Lardy, 1972a; Wong, Wilkinson, Hamill & Horng, 1973), with consequent reduction of the energy supply needed to maintain ionic gradiants, and (2) a rise in the cytoplasmic concentration of calcium ions by transfer of calcium from extracellular or intracellular sources (Caswell & Pressman, 1972; Reed & Lardy, 1972b; Scarpa, Baldassare & Insei, 1972). A rise in intracellular calcium has been reported to depolarize squid axon (Grundfest, Kao & Altamirano, 1954; Hodgkin & Keynes, 1956).

How the ionophores depolarize and why X-537A is more effective than A23187 is of less concern to us here than the functional consequences of this pattern. It is evident that the relatively strong depolarizing effect of X-537A could account for its greater effectiveness in eliciting secretion from neurohypophyseal terminals and chromaffin cells as earlier suspected (Nakazato & Douglas, 1974; Cochrane *et al.*, 1975): depolarization, in the presence of calcium, provides an adequate stimulus for secretion in both these preparations. Depolarization could also, for the same reason, account for the strong effects of X-537A on transmitter release from cholinergic (Kita & van der Kloot, 1974) and adrenergic neurones (Thoa, Costa, Moss & Kopin, 1974). The fibrillation we observed in muscles exposed to X-537A could be due to release of acetylcholine from motor nerve terminals while the absence of fibrillation in response to A23187 may reflect its inability to adequately depolarize these terminals.

The finding that A23187 depolarized more rapidly when calcium was omitted from the bathing medium may explain the contrasting effects of this ionophore on isolated neurohypophyses in different experiments. Nakazato & Douglas (1974) found that A23187 had little or no stimulant effect on neurohypophyses bathed throughout with a calcium-containing solution. Russell, Hansen & Thorn (1974), on the other hand, noted a sharp rise in vasopressin output when calcium was added to neurohypophyses bathed for 70 min in a calcium-free medium with A23187 present for the final 40 minutes. Under these conditions the neurohypophyseal terminals may well have been depolarized. This once more raises the possibility (Douglas, 1968) that calcium influx alone may not provide an optimal stimulus for secretion. Clearly, depolarizing actions, and the changes in membrane properties they involve, must be considered as possible factors in functional responses to these ionophores. It remains to be seen whether the damage to the fibres we observed after prolonged exposure to the ionophores indicate a general action on cell membranes or an effect peculiar to muscle fibres that may arise from intense contraction.

We thank Dr M.W. Osborne of Hoffman-La Roche, Inc. and Dr R.L. Hamill of Eli Lilly and Co. for generous gifts of X-537A and A23187 respectively. D.E.C. is a NIH Postdoctoral Fellow. This work was supported by a grant from the USPHS.

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(Received February 26, 1975)

Br. J. Pharmac. (1975), 54, 403-405

In vitro INHIBITION OF BRAIN MITOCHONDRIAL MONOAMINE OXIDASE BY 6-HYDROXYDOPAMINE

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6-Hydroxydopamine (6-OHDA) inhibits rat brain mitochondrial monoamine oxidase (MAO) when kynuramine or dopamine are used as substrates. The effect is competitive and reversible giving *Ki* values of 74 and 176 μM with the respective substrates At high concentrations (5 mM) of each substrate, inhibition of MAO was not observed.

Introduction 6-Hydroxydopamine (6-OHDA) produces a relatively selective degeneration of adrenergic nerve terminals (see Kostrzewa & Jacobowitz, 1974 for review). Although the precise mechanism by which it acts is not known, the highly reactive products e.g. hydrogen peroxide, super-oxide and quinones generated during the reaction may react nonspecifically with neuronal structures, eventually destroying them (Heikkila & Cohen, 1971, 1972, 1973). 6-OHDA is a substrate for monoamine oxidase (MAO) (Malmfors & Sachs, 1968, Malmfors, 1971; Jonsson, Malmfors & Sachs, 1972). This enzyme, together with granule storage, may thus serve as protective mechanisms (Kostrzewa & Jacobowitz, 1974). Recently Agid, Javoy & Youdim (1973) showed that injection of 6-OHDA (20 μg) directly into the substantia nigra brings about a decrease in activity of MAO as well as of aldehyde dehydrogenase, although such an effect may be nonspecific (Duncan, Sourkes, Duborovsky & Quik, 1975). The present study was undertaken to examine whether 6-OHDA inhibits MAO activity *in vitro.*

Methods Male Charles River rats (250-300 g body weight) were killed by decapitation. The brains were rapidly removed and homogenized in ice-cold 0.32 M sucrose solution with a Teflon pestle homogenizer to give a final 10% (w/v) preparation. Crude brain mitochondria were prepared by the method of Hawkins (1952). MAO activity was assayed using kynuramine as substrate by the spectrofluorimetric procedure of Kraml (1965). The enzyme mixture in a total volume of 1 ml contained 100 μl of 10% (w/v) crude brain mitochondria, 500 μl 0.10 M phosphate buffer (pH 7.4), 100 μl of 1 mM kynuramine and water. The reaction which was linear for the first 20 min was terminated by the addition of 500 μl 10% (w/v) trichloroacetic acid, and the 4-hydroxyquinoline formed estimated fluorimetrically (Kraml, 1965). When dopamine was the substrate, MAO activity was measured by the procedure of Robinson, Lovenberg, Keiser & Sjoerdsma (1968).

Results When kynuramine concentration was 0.1 mM, the addition of 6-OHDA caused inhibition of MAO activity. The degree of inhibition was 15, 30, 42 and 82% when 6-OHDA concentrations were 0 001, 0.01, 0.1 and 1.0 mM respectively. Preincubation of enzyme preparation for 30 min at 37° C with these concentrations of 6-OHDA prior to the addition of kynuramine resulted in a slight increase in inhibitory effect, the values being 25, 42, 50 and 87% respectively.

When these results were plotted according to the method of Lineweaver & Burk (1934) and Dixon (1953) the results shown in Figure 1a and b were obtained. The graphs demonstrate a reversible and competitive inhibition of oxidative deamination of kynuramine by 6-OHDA with a *Ki* value of 74 μM which is similar to the *Km* for kynuramine oxidation by MAO (35-85 μM) (Youdim, Collins & Sandler, 1969) The deamination of dopamine was inhibited in a similar way by 6-OHDA, the *Ki* value being 176 μM which is close to the *Km* (160 μM) for dopamine (Achee, Togulga & Gabay, 1974).

At high concentrations of substrate (5 mM) there was no inhibition of MAO by 0.1 mM 6-OHDA as would be expected from the nature of the inhibition. This concentration is 10 times lower than that used by Wagner & Trendelenburg (1971).

Discussion The present study clearly demonstrates inhibition of MAO by 6-OHDA when kynuramine or dopamine are used as substrates This effect is reversible and competitive with a *Ki* value similar to the *Km* for its substrates. Wagner & Trendelenburg (1971) reported that there was no inhibition of liver homogenate MAO by 6-OHDA. Their results can be simply explained by the fact that they used substrate concentrations of

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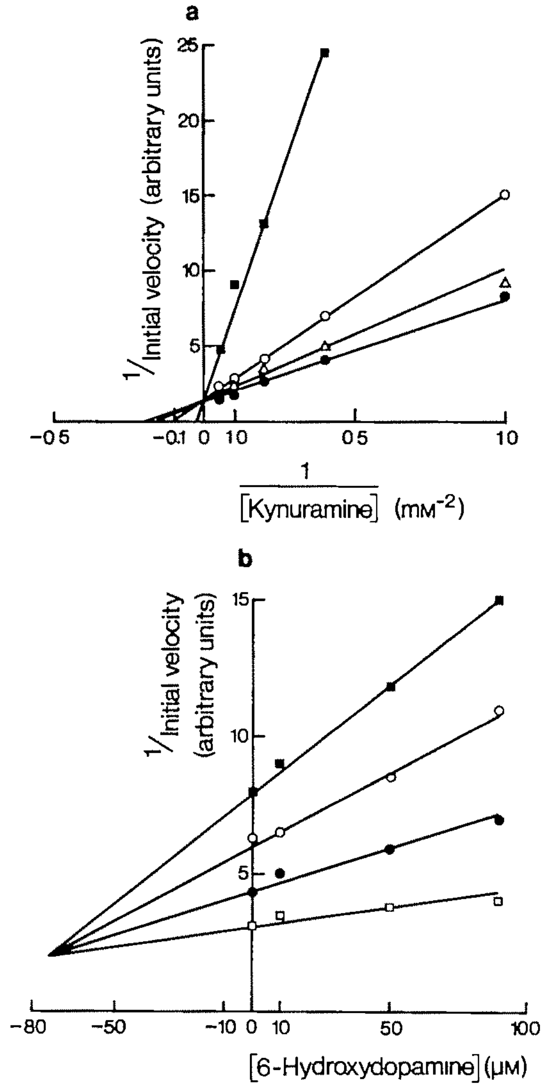

Figure 1 (a) Reciprocal plots of the inhibition of monoamine oxidase (MAO) by 6-hydroxydopamine (6-OHDA). Reciprocal plots of initial velocities against kynuramine concentration at different 6-OHDA concentrations: zero (●), 0.01 mM (△), 0.1 mM (○) and 1 mM (■). (b) Dixon (1953) plots of the inhibition of MAO by 6-OHDA. Reciprocal plots of initial velocities against inhibitor concentrations at a series of kynuramine concentrations: 0.01 mM (■), 0.025 mM (○), 0.05 mM (●) and 0.1 mM (□).

50 mM, some five hundred times greater than the enzyme *Km* (see Tipton, 1968; Tipton & Spires, 1971).

Previous studies have indicated that 6-OHDA is actively accumulated by catecholaminergic neurones where it is localized in granules, eventually producing degenerative effects (Kostrzewa & Jacobowitz, 1974). The initial action of 6-OHDA is not known, although it does uncouple oxidative phosphorylation in concentrations which have been calculated to occur intraneuronally (Wagner & Trendelenburg, 1971) and cause a loss in ability to conduct action potentials (Haeusler, 1971). 6-OHDA is a substrate for MAO (Senoh, Creveling, Udenfriend & Witkop, 1959; Malmfors, 1971), and MAO inhibitor pretreatment potentiates its effect on adrenergic neurones (Malmfors, 1971, Jonsson *et al.*, 1972). At concentrations (0.05 mM) sufficient to uncouple oxidative phosphorylation (Wagner & Trendelenburg, 1971), it inhibits MAO by about 60%. The inhibition which is competitive can be produced by 6-OHDA itself, by the hydrogen peroxide generated from its action or by both (Heikkila & Cohen, 1971, 1973).

It has already been shown that hydrogen peroxide is able to inhibit brain MAO activity competitively (Tipton, 1968; Tipton & Spires, 1971). Thus it is possible that concentrations of 6-OHDA used for selective degeneration of adrenergic neurones in the central nervous system would be high enough to inhibit intraneuronal as well as extraneuronal MAO reversibly. Furthermore, it is probable that the degree of MAO inhibition is even greater intraneuronally since 6-OHDA can be taken up and accumulated within the adrenergic neurone. It is tempting to speculate that initial reversible inhibition of MAO may be involved in the events leading to the degeneration of adrenergic nerve endings after administration of 6-OHDA.

This work was partly supported by a Travelling Fellowship from the Wellcome Trust and INSERM which is gratefully acknowledged. I thank Dr J. Glowinski in whose laboratory this work was performed.

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(Received March 18, 1975)

Br. J. Pharmac. (1975), 54, 406-408

THE γ-AMINOBUTYRIC ACID SYSTEM IN RAT CEREBELLUM DURING CANNABINOID-INDUCED CATALEPTOID STATE

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Repeated, but not single, intraperitoneal injections of $\Delta^{1,6}$-tetrahydrocannabinol ($\Delta^{1,6}$-THC) 20 mg/kg to rats administered daily for two weeks, produced increased γ-aminobutyric acid (GABA) concentration and decreased glutamic acid decarboxylase (GAD) activity in the cerebellum, as well as enhancement of [^{3}H]-GABA uptake by cerebellar crude synaptosomes. It seems that the motor impairment elicited by $\Delta^{1,6}$-THC was not associated with the GABA system, but presumably might be related to changes in brain excitability.

Introduction Severe motor disturbances appeared in humans (Paton & Pertwee, 1973) and animals (Grunfeld & Edery, 1969) following administration of psychopharmacologically active cannabinoids. The mechanism(s) responsible for these motor impairments have not yet been elucidated. The cerebellum is considered to play an important role in motor control and maintenance of body equilibrium. The action of the cerebellum is mainly inhibitory, shown to be mediated by γ-aminobutyric acid (GABA) in interneurones and Purkinje cells (Kuriyama, Haber, Sisken & Roberts, 1966; Obata & Takeda, 1969).

The purpose of this work was to examine whether the GABA system, i.e., the concentration of GABA, its synthesis by glutamic acid decarboxylase (GAD), or the uptake of GABA by nerve terminals was affected during the cannabinoid-induced cataleptoid state in rats.

Part of the present results was communicated at the 32nd meeting of the Israel Physiological and Pharmacological Society, in February, 1975.

Methods Albino rats of either sex, 170-200 g, were injected intraperitoneally with 20 mg/kg of $\Delta^{1,6}$-tetrahydrocannabinol ($\Delta^{1,6}$-THC) dissolved in propylene glycol. Control animals were injected with the vehicle only. $\Delta^{1,6}$-THC was administered either as one single dose or as repeated single daily doses for 2 weeks. In all cases the maximum volume injected was 0.1 ml/100 g body weight. The rats were killed at the peak of motor disturbances elicited by $\Delta^{1,6}$-THC and the cerebellum was quickly removed. In some experiments brain regions associated with motor control such as the cerebellar flocculus and anterior vermis as well as the corpus striatum and parieto-temporal cerebral cortex were dissected. For GABA determination (Strasberg & Elliott, 1967) half of the cerebellum was immediately frozen in liquid N_2 and the remaining half was rapidly homogenized for subsequent GAD assay (Roberts & Simonsen, 1963). GABA uptake by crude cerebellar synaptosomes was determined by the method described by Iversen & Johnston (1971), with slight modifications. After 5 min incubation at 25° C, the [^{3}H]-GABA-containing particles were sedimented by high-speed centrifugation, rinsed with cold 0.9% w/v NaCl solution (saline) and recentrifuged. The pellet was dissolved with 0.3 ml of the tissue solubilizing agent NCS (Amersham) and the radioactivity was counted in toluene containing 0.5% w/v PPO (2,5-diphenyloxazole) and 0.03% w/v dimethyl POPOP [1,4-di-(2(5-phenyloxazole))-benzene]. The uptake was calculated as tissue : medium ratio and corrected for zero time, blank samples.

Results Following administration of 20 mg/kg of $\Delta^{1,6}$-THC severe cataleptoid reaction appeared in all animals within 30 min and normal behaviour returned 2 h later. The cataleptoid reaction was less severe towards the end of the period of repeated daily injections. In addition, there was a gradual decrease of body weight in the $\Delta^{1,6}$-THC treated group, but not in the control. The rats regained their normal weight by the end of the second week.

A single injection of $\Delta^{1,6}$-THC produced no changes in whole cerebellar GABA content or synthesis (Table 1). Similarly, no changes were found in discrete areas such as the flocculus and anterior vermis, as well as in the corpus striatum and cerebral cortex. In contrast, repeated daily treatment induced an increase in GABA concentration and a decrease in GAD activity. A small, but

Table 1 Effect of $\Delta^{1,6}$-tetrahydrocannabinol ($\Delta^{1,6}$-THC) on γ-aminobutyric acid (GABA) concentration, glutamic acid decarboxylase (GAD) activity and [^{3}H]-GABA uptake in rat cerebellum.

	Treatment	*GABA (μmol/g)*	*GAD (μmol/g^{-1} h^{-1})*	*[^{3}H]-GABA uptake Tissue: medium ratio* §
Single injection†	Control (propylene glycol)	1.49 ± 0.09	16.67 ± 0.60	
	$\Delta^{1,6}$-THC (20 mg/kg)	1.59 ± 0.06	16.21 ± 0.23	
Repeated single daily injections (2 weeks)†	Control (propylene glycol)	1.34 ± 0.07	17.66 ± 0.60	3.11 ± 0.25
	$\Delta^{1,6}$-THC (20 mg/kg)	2.21 ± 0.26*	13.31 ± 0.60**	4.98 ± 0.28**

§ Tissue : medium ratio expresses the amount of [^{3}H]-GABA taken up by the crude cerebellar synaptosomes compared to that in the medium (ct/min in 1g of original tissue : ct/min in 1 ml of incubation medium)
† 0.1 ml/100 g body weight.
Results are mean ± s.e. of six rats. * $P < 0.05$; ** $P < 0.001$ (Student's *t*-test).

significant, drop in GAD activity was also found in the motor cortex of these animals (15.04 ± 0.39 μmol g^{-1}h^{-1}; 6 rats; $P < 0.05$) compared to control (17.99 ± 1.36; 6 rats). No change of GAD activity was observed in the corpus striatum.

The uptake of [^{3}H]-GABA by cerebellar crude synaptosomes was increased after 2 weeks of daily injections of $\Delta^{1,6}$-THC. However, no such effect was observed when the rats were killed 3 days after the last injection (tissue : medium, 2.98 : 3.12; 2 rats).

Discussion The present results indicate that motor disturbances in rats produced by either single or repeated injections of $\Delta^{1,6}$-THC could not be ascribed to changes in GABA concentration or synthesis in discrete brain regions. Leonard (1971) found a slight decrease (9-12%) in GABA in whole brain of rats during severe cataleptoid state produced by single doses (50 mg/kg and 100 mg/kg) of $\Delta^{1,6}$-THC. Comparison of the results is difficult since Leonard used much higher doses than those used in the present work and there was no indication whether precautions were taken to prevent post-mortem changes in GABA content (Norberg & Siesjö, 1975).

The uptake of GABA into nerve terminals was found to be decreased following ATPase inhibition (Iversen & Neal, 1968). On the contrary, increased ATPase activity caused by cannabinoids (Jain, Curtis & Bakutis, 1974) could be responsible for the enhancement of GABA uptake observed in this work. The last phenomenon could in turn result firstly in increased GABA concentration, and secondly in decreased GAD activity. The latter has been shown to be regulated by a feedback mechanism involving repression of the enzyme activity *pari passu* with GABA accumulation in nerve terminals (Haber, Sze, Kuriyama & Roberts, 1970).

The observed changes in the cerebellar GABA system were not related to the motor disturbances. On the other hand, they might be associated with the electrophysiological changes such as high voltage waves intermingled with epileptiform bursts elicited by cannabinoids in the cerebellum (Martinez, Stadnicki & Schaeppi, 1972), presumably reflecting alterations of neuronal activity. In this regard, Elliott (1965) postulated that free but not stored GABA could affect neuronal function.

The able technical assistance of Mr N. Oz is gratefully acknowledged. Special thanks to Mrs Malka Gottesfeld for her valuable assistance. Please send reprint requests to Z.G.

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(Received April 18, 1975)

Br. J. Pharmac. (1975), 54. 409-410

EXTRACTION FROM OX RETRACTOR PENIS OF AN INHIBITORY SUBSTANCE WHICH MIMICS ITS ATROPINE-RESISTANT NEUROGENIC RELAXATION

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The inhibitory post-ganglionic transmission in the retractor penis of the ox resembles that of the dog and is not cholinergic or adrenergic. Acid extracts of this tissue have yielded an unidentified, labile, inhibitory substance which mimics the effect of its inhibitory nerves.

Introduction

The retractor penis (R.P.) of the ox is known to be free from skeletal fibres and from ganglion-cells (Klinge, Pohto & Solatunturi, 1970) We find that it has the advantage, over that of other species (pig, sheep, horse etc.), of developing tone spontaneously in Krebs-Henseleit solution containing phentolamine, without requiring drug-induced contraction (by guanethidine or by spasmogens) to reveal the effect of its inhibitory innervation. Our results also show that this smooth muscle contains post-ganglionic inhibitory nerves with atropine-resistant transmission, which resemble the parasympathetic sacral innervation of the dog R.P., first studied by Langley & Anderson (1895) and shown by Luduena & Grigas (1966, 1972) to be non-cholinergic and not purinergic. Extracts of the R.P. tissue contain an unidentified, labile, inhibitory substance which could account for this type of transmission

Methods

Isolated preparations. Steer R.P., obtained from various abattoirs, were transported at 0° C. Strips, 30 x 2 x 2mm, were dissected longtitudinally, usually from the middle third of the R.P. and suspended at 35° C in Krebs-Henseleit solution (mM. NaCl 112.9, KCl 4 69, $CaCl_2.2H_2O$ 2.52, KH_2PO_4 1.18, $MgSO_4.7H_2O$ 1.1 or 1.5, $NaHCO_3$ 25.0 and glucose 11.1) containing phentolamine, 5.3 µM. Contractions were recorded isometrically via transducers at a load of 2 grams The preparations were stimulated transmurally at a pulse width of 1 ms, usually with single pulses at 1-2 min intervals. Some preparations, left over night in the organ bath at room temperature, could be used again at 35° C next morning, when they often appeared to be more sensitive to extracts. Drug concentrations are expressed as their respective salts.

Extraction of inhibitory substance(s). Two to six hours after death, 1 g pieces of R.P from the proximal or middle third were solidified in liquid N_2, hammered in N_2-cooled, stainless steel or PTFE pulverizers, ground with disodium edetate (EDTA)-washed Pyrex fragments in ice-cooled Pyrex mortars containing 0.17 or 0.33 N HCl ('Aristar' in distilled water, deionized through Bio-Rad resin AG 501-X8), 1 ml/100 or 200 mg tissue, and centrifuged (0° C, 16000 *g*, 10 minutes). The ice-cooled supernatants, of pH 1-2, were titrated electrometrically to pH 6-7 with cold 'Aristar' 2 N NaOH or 'Analar' 1 N Na_2CO_3, sometimes in the presence of ca. 1/40 vol of sodium acetate buffer (0.2 M; pH 3) and were re-centrifuged, or allowed to sediment. Their inhibitory activity was determined immediately on the more sensitive of twin steer R.P. preparations in the presence of atropine, phentolamine propranolol and mepyramine.

Results

Pharmacological analysis of the inhibitory transmission. The isolated preparations at first lengthened; acetylcholine chloride, 1-10 µg/ml (cf. Klinge, 1970), 5-hydroxytryptamine creatinine sulphate, 10 µg/ml, histamine dihydrochloride, 1-10 µg/ml and adenosine 5'-triphosphate (ATP) disodium salt, 10 µg/ml, were motor

After 0.5-4 h tone (≥ 2 g) appeared, which was usually well maintained; a few preparations in which the tone level was not steady were discarded. As soon as tone developed, single 1 ms

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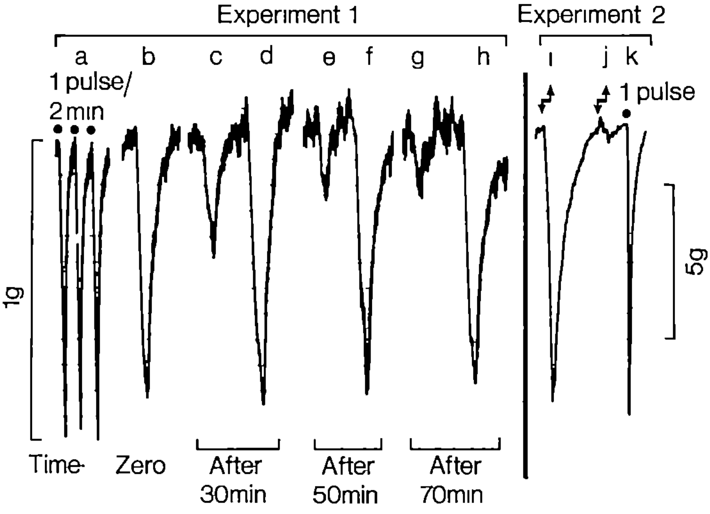

Figure 1 The inhibitory activity of steer retractor penis (R.P.) extracts decays at room temperature (b-h) and is destroyed by heat ((i-k), from another experiment). Both assays on tonic steer R.P strips in phentolamine, 5.3 μM, mepyramine, 1 25 μM, propranolol, 6.8 μM for Expt. 1 and 13.6 μM for Expt. 2 and atropine, 1.44 μM for Expt. 1 and 2.88 μM for Expt. 2. Neurogenic relaxations elicited by single 1 ms pulses at the dots in (a) and (k) are matched by inhibitory responses to R.P. extracts, given for 1 minute. (b-h), Responses to a fixed dose, ≡ 1 mg of tissue/ml, of an extract of pH 6 1. at (b), (d), (f) and (h), control sample of the extract kept on ice, at (c), (e) and (g), parallel sample kept at 23° C, showing decay after 30 (c), 50 (e) and 70 (g) minutes. (i and j), Thermolability of another R P extract, pH 6.6· ≡ 2 5 mg of tissue/ml before (i) and after (j) heating to 100° C for 2 minutes.

pulses elicited large, tetrodotoxin-susceptible inhibitions which were non-cholinergic because they were not mimicked by acetylcholine nor influenced by physostigmine, atropine, dimethyltubocurarine or pentolinium. These inhibitory responses were unaffected by guanethidine, propranolol, mepyramine or burimamide, and were not mimicked by noradrenaline adrenaline, dopamine, 5-hydroxytryptamine, various amino acids (glycine, alanine, glutamic acid, aspartic acid and γ-aminobutyric acid) or by ATP.

Inhibitory activity of R.P extracts. Unlike parallel, cold reagent blanks, the steer R.P extracts, in doses of ≡ 1 5 mg tissue/ml, produced relaxations, sometimes quite marked (Figure 1), which mimicked the neurogenic inhibitions and persisted in tetrodotoxin, dimethyltubocurarine or burimamide. Partitioned at pH < 2 with 2-10 vol of freshly distilled ether, this inhibitory activity remained in the aqueous phase and was therefore not due to prostaglandins.

The lability of this inhibitory activity was shown by the fact that it decayed (more rapidly at room temperature) and could be destroyed by 2-5 min boiling (Figure 1), presumably because of this lability several extracts have had poor activity.

We wish to express our thanks to Mr J. Verney and to Mr N. Gillespie for valuable help.

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(Received May 13, 1975)

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397 SHORT COMMUNICATIONS

Printed in Great Britain by The Whitefriars Press Ltd, London and Tonbridge

~~CURRENT ISSUE~~

British Journal of Pharmacology

August 1975

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The British Journal of Pharmacology is published monthly by Macmillan Journals Ltd, 4 Little Essex Street, London WC2R 3LF. Telephone: 01-836 6633.

Annual subscription £36.00 ($90 USA and Canada). (Orders must be accompanied by remittance. Prices applicable only to orders received before 31st December 1975.)

Cheques should be made payable to Macmillan Journals Limited: Subscription Department, Brunel Road, Basingstoke, Hampshire RG21 2XS, England.

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Second class postage paid at New York, N.Y.
US Mailing Agent, Air and Sea Freight Inc., 527 Madison Avenue, New York, NY 10022.

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Systematic Pharmacology

SP1	Cardiovascular system
SP2	Central nervous system
SP3	Other physiological systems

Pharmacokinetics

PK1	Storage, distribution, fate
PK2	Synthesis, metabolism
PK3	Uptake, release

CP	**Clinical Pharmacology, Experimental Therapy**
CT	**Chemotherapy**
M	**Miscellaneous**

Autopharmacology

AP1	Adrenergic, cholinergic transmission
AP2	Histamine, kinins, prostaglandins, etc.
AP3	Hormones

Drug Mechanisms

DM1	Molecular, cellular, biochemical, biophysical
DM2	Receptors, antagonism, potentiation, tolerance

PM	**Pharmacometrics**
PP	**Psychopharmacology**
TX	**Toxicology**

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THE EFFECT OF ANOXIA ON THE VENTRICULAR FIBRILLATION THRESHOLD IN THE RABBIT ISOLATED HEART

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1 The ventricular fibrillation threshold (VFT) was measured in the isolated heart of the rabbit perfused via the aorta with McEwen's solution at 37° C by applying a single 10 ms pulse of current during the vulnerable period of late systole. The arrhythmia induced was either fibrillation or a rapid tachycardia.

2 Gassing the McEwen's solution with 5% CO_2 in N_2 (anoxia) instead of with carbogen caused a negative inotropic and chronotropic effect and significantly lowered the VFT. Although anoxia releases noradrenaline from the heart the effect of anoxia on the VFT was not prevented by β-adrenoceptor blockade with propranolol or pindolol or by previous treatment with reserpine.

3 Perfusion with adenosine (5 μM) which is released from the heart muscle by anoxia, or with dipyridamole (10 μM) which protects the adenosine from binding or destruction by the tissues, or with both combined failed to alter the VFT significantly. Furthermore neither adenosine nor dipyridamole significantly altered the effect of anoxia on the VFT.

4 Anoxia, adenosine and dipyridamole significantly increased the duration of the induced arrhythmia when compared with that of the controls.

5 Anoxia and adenosine significantly shortened the vulnerable time, i.e. the minimal time after the R-wave of the ECG at which the pulse had to be applied to induce the arrhythmia.

6 Perfusion with the McEwen's solution gassed with 5% CO_2 in air (hypoxia) significantly lowered the VFT but the effect was not as great as with anoxia. Isoprenaline when infused lowered the VFT but this effect was not potentiated by hypoxia.

7 The results indicate that (a) anoxia lowers the VFT in the perfused isolated heart of the rabbit and that this effect is not due to adenosine or noradrenaline released by the anoxia and (b) hypoxia does not sensitize the heart to the arrhythmic effect of isoprenaline.

Introduction

There is a considerable body of undisputed evidence that a localized area of ischaemia with a resultant anoxia of that part of the heart muscle produced experimentally by occlusion of a coronary artery in animals (Harris, 1948; Chardack, Gage, Frederico, Cusick, Matsumoto & Lanphier, 1964) or occurring spontaneously in man (Miller, 1939; Smith, 1939) gives rise to ventricular arrhythmias including fibrillation and also lowers the ventricular fibrillation threshold (VFT) of the infarcted area (Wiggers, Wégria & Pinera, 1940; Shumway, Johnson & Stish, 1957; Han, 1969). However, the experimental results obtained where there is a generalized hypoxia of the animal are contradictory. Coffman & Gregg (1960) showed that asphyxia induced by tracheal occlusion produced ventricular fibrillation in one third of their dogs and Szekeres & Papp (1967) found that ventilation with 5-10% O_2 lowered the VFT in the cat. On the other hand Badeer & Howarth (1959) showed that ventilation with 100% N_2 failed to induce ventricular fibrillation in dogs and Turnbull, MacLean, Dobell & Demers (1965) ventilating dogs with 5-10% O_2 and Rogers, Spear, Moore, Horowitz & Sonne (1973) using 10-16% O_2 could detect no significant change produced on the VFT. These results appear to confirm the suggestion that a boundary or trigger zone between normal and hypoxic myocardium was important for initiating and maintaining cardiac arrhythmias (Harris, 1948). However, Badeer & Howarth (1959) showed that when both coronary arteries were ligated so that apparently all the myocardium was rendered ischaemic, ventricular fibrillation occurred spontaneously in 9 out of 10 dogs.

In all of these studies on the whole animal the effect of O_2 lack could have been influenced by extracardiac factors, e.g. release of catecholamines. In order to determine the direct effect of O_2 lack on the heart it is therefore desirable to use an isolated heart preparation. Szekeres & Papp (1967) found that when the heart-lung preparation of the cat was ventilated with 5% O_2 the VFT was raised. They ascribed this to lack of neural and hormonal influences.

The purpose of the present investigation was to study the effect of O_2 lack on the isolated Ringer-perfused heart of the rabbit. Burn & Huković (1960) had demonstrated that hypoxia predisposed to a persistent fibrillation but they did not measure the minimal current which was required to induce it. In this present study the effect of O_2 lack on both the VFT and the duration of the arrhythmia has been measured. In addition, attempts have been made to elucidate the mechanism by which lack of O_2 may predispose to cardiac arrhythmias.

Methods

Ventricular fibrillation or a rapid tachycardia was induced in rabbit hearts perfused via the aorta with McEwen's (1956) solution at 37°C by the method described previously (MacConaill & Murnaghan, 1967). The fibrillation threshold was determined by measuring the minimal strength needed to produce arrhythmia of a 10 ms square-wave pulse of current applied after each 8th heart beat during the vulnerable period of late systole. It was achieved by testing at 5 ms steps during the latter half of the R-T interval. The minimal acceptable duration of the induced arrhythmia was set at 1 s because of difficulty in differentiating it from groups of 3 or 4 multiple extrasystoles.

The type of arhythmia induced, monitored on a large oscilloscope screen (Airmec), was recorded as fibrillation or tachycardia. The duration of the arrhythmia was listed as persistent ($>$ 60 s) or nonpersistent. If a normal rhythm had not returned after 60 s, defibrillation was effected by manually infusing 0.3 M KCl (usually 0.5 ml sufficed) into the aortic cannula. The term ventricular fibrillation threshold (VFT) was used to indicate the minimal current required to induce either fibrillation or tachycardia. In order to indicate the magnitude of change produced by the treatment on the VFT, despite the variation in magnitude of the latter among hearts, the VFT change ratio was calculated, i.e. VFT during treatment/VFT of the preceding control(s). If a drug was combined with anoxia or hypoxia then the former administered alone acted as control. The minimal time after the R wave of the ECG at which the 10 ms pulse had to be applied to induce the arrhythmia was called the vulnerable time.

The perfusion apparatus consisted of three heat-exchange columns connected to a perspex block fitted with a tap so that the fluid from any one column could be selected. Each column was fed from a reservoir bottle; the control perfusion fluid was gassed with 5% CO_2 in O_2. In order to expose the heart to anoxia the perfusion fluid was gassed with 5% CO_2 in N_2, for hypoxia 5% CO_2 in compressed air was used. At the front of the block an opening, with tap, permitted the injection of KCl into the perfusion fluid just above the aortic cannula; at the back of the block was an opening, with tap, for removal of air bubbles from the cannula when required. Adenosine and dipyridamole when used separately were incorporated in the perfusion fluid; when combined the adenosine was dissolved in 160 mM sodium chloride and infused by a syringe pump at a constant rate into the aortic cannula via an opening on the side of the perspex block. Dipyridamole was dissolved in 0.1 M HCl and then diluted approximately 8000 times with McEwen's solution. Isoprenaline was dissolved in 160 mM sodium chloride acidified with HCl to pH5 and infused with the syringe pump at a rate of 3.2 nmol/minute. Reserpine 1 mg/kg was injected intraperitoneally 48 and 24 h before the rabbit was killed.

Perfusion with solutions deficient in O_2 was rarely maintained for a period longer than 5 min in order to avoid undue depression of the heart. If the VFT had not been determined by the end of this anoxic or hypoxic period the heart was perfused with oxygenated McEwen's solution before testing again at higher current strengths with the O_2-deficient solution. When dipyridamole, propranolol or pindolol were used they invariably were perfused for at least 10 min in oxygenated McEwen's solution before being perfused in an O_2-deficient medium. Perfusion with adenosine and isoprenaline commenced simultaneously with the period of exposure to O_2 lack.

The results are usually expressed by the arithmetic mean with its standard error. Significance of difference was determined by Student's t test or the Chi-square test where appropriate.

Drugs used were: adenosine (B.D.H.), dipyridamole (Persantin, Boehringer Ingelheim Ltd.), propranolol (ICI), pindolol (Sandoz Products Ltd.), isoprenaline hydrochloride (Winthrop) and reserpine (Koch-Light Lab.).

Results

Throughout the course of perfusion of the heart with the McEwen's solution, the force of contraction decreased more than the coronary flow. In the 16 hearts in the first trial with anoxic McEwen's solution (i.e. gassed with 5% CO_2 in N_2) at 132 ± 13 min after the start of the perfusion, the preceding control systolic force was 12.6 ± 3.2 g and the coronary flow 16.2 ± 1.2 ml/minute. On the last anoxic trial at 329 ± 23 min they were 1.2 ± 0.3 g and 9.6 ± 1.0 ml/min respectively.

At the end of the period of perfusion with the anoxic McEwen's solution in 53 trials the heart rate had decreased by 84 ± 7 beats/min from the preceding control value of 185 ± 6, the force of contraction had decreased 60 ± 3% and the coronary flow had increased. The magnitude of the negative inotropic effect was independent of the degree of contractility preceding the period of anoxia. However, the increase in coronary flow was clearly related to the control contractility of the heart. Early in the experiment when the latter was 10.6 ± 0.5 g (20 trials) the increase in the coronary flow was 86 ± 15.5%; later when 3.5 ± 0.6 g (19 trials) it was 45 ± 9% and near the end of the experiment when 1.3 ± 0.2 g (10 trials) it was only 12 ± 4%. Adenosine and dipyridamole when incorporated in the perfusion fluid increased the coronary flow.

In 6 trials the effect of anoxia on the Q-T interval was measured. Anoxia significantly ($P < 0.001$) shortened the interval by 44 ± 6 ms from a control value of 131 ± 4 ms despite a significant ($P < 0.02$) decrease in heart rate from 167 ± 13 to 117 ± 11 beats/minute. In 6 trials adenosine (5 μM) alone and in 2 trials dipyridamole (10 μM) alone had no significant effect on the Q-T interval. Dipyridamole when combined with adenosine (4 trials) significantly ($P < 0.01$) shortened the Q-T interval from 136.3 ± 2.4 to 118.8 ± 3.2 milliseconds.

Ventricular fibrillation threshold

In 32 out of 40 trials in 14 rabbit hearts the ventricular fibrillation threshold (VFT) was lowered during the period of anoxia (Table 1). The total duration of the period of applied anoxia was 4.5 ± 0.44 min; the time from its start until the arrhythmia was induced was 2.5 ± 0.26 minutes. The VFT varied between 2.5-63 mA in the controls and 1-16 mA during anoxia. In order to indicate the magnitude of change produced by anoxia on the VFT despite the variation in magnitude of the control values among hearts, the VFT change ratio was calculated, i.e. the VFT during anoxia/VFT of the preceding control(s). The mean ± s.e. mean VFT change ratio for all 40 trials was 0.60 ± 0.05 (range 0.14–1.28) which was significantly different from unity ($P < 0.001$). There was an indication in the results that if the anoxia was prolonged it was less likely to lower the VFT.

During anoxia of the heart muscle adenosine has been shown to be liberated and is believed by some workers (Berne, 1964; Rubio, Berne & Katori, 1969) to account for the coronary vessel dilatation. The possibility that this mechanism could account for or influence the change in the VFT produced by anoxia had to be considered. However, when incorporated in the McEwen's solution adenosine (5 μM) alone (20 trials), or 10 μM dipyridamole alone (24 trials), which protects the former from destruction (Berne, 1966), or both combined (2 trials) failed to alter significantly the VFT change ratio (0.82 ± 0.09,

Table 1 Effect of lack of oxygen and of drugs on induced ventricular fibrillation

Treatment	*No. of hearts*	*Proportion of trials VFT was reduced*	*VFT change ratio. Mean ± s.e. mean*	*Value*[*] P<
Anoxia	14	32/40	0.60 ± 0.05	0.001
Dipyridamole 10 μM	6	16/24	0.86 ± 0.10	NS
Adenosine 5 μM	3	14/20	0.82 ± 0.09	NS
Dipyridamole + adenosine	1	0/2	1.30 ± 0.30	NS
Anoxia + dipyridamole	6	9/13	0.83 ± 0.06	0.02 (NS)
Anoxia + adenosine	1	3/3	0.66 ± 0.10	NS (NS)
β-blockade	2	9/15	0.95 ± 0.11	NS
Anoxia + β-blockade	2	7/7	0.66 ± 0.07	0.01 (NS)
Anoxia + reserpine treatment	1	3/3	0.41 ± 0.06	0.02
Hypoxia	5	11/15	0.75 ± 0.07	0.01
Hypoxia + isoprenaline	2	5/5	0.60 ± 0.09	0.02 (NS)
Isoprenaline	2	3/3	0.59 ± 0.04	0.02

[*] Significance from unity and in parentheses comparison with O_2 lack alone restricted to tests on the same hearts.

0.86 ± 0.10 and 1.30 ± 0.30 respectively). Furthermore the fall in the VFT produced by anoxia during perfusion with $10 \mu M$ dipyridamole (0.83 ± 0.06) in 13 trials was not significantly different from that produced in 17 trials by anoxia alone (0.82 ± 0.06) on the same 6 rabbit hearts but each VFT change ratio was significantly different from unity ($P < 0.02$ and 0.01 respectively). Finally adenosine ($5 \mu M$) in 3 trials did not significantly alter the response to anoxia (0.66 ± 0.10) compared with 4 trials of anoxia alone on the same two hearts (0.67 ± 0.14). However, neither mean was significantly different from unity probably because of the small number of values in each case.

During anoxia noradrenaline is liberated from the rabbit isolated perfused heart (Wollenberger & Shahab, 1965) and when infused into it has been shown to lower the VFT (Murnaghan, 1975). However, this mechanism does not appear to be responsible for the fall in the VFT during anoxia because when the β-adrenoceptors were blocked with propranolol ($2 \mu M$) or pindolol in 7 trials the VFT was still significantly reduced by anoxia (0.66 ± 0.07, $P < 0.01$). Additional evidence that the release of noradrenaline is not responsible for the fall in the VFT during anoxia is the fact that the fall was not prevented by previous treatment with reserpine (VFT change ratios of 0.44, 0.50 and 0.29 in three trials in one heart). The β-blockers alone in 15 trials did not significantly alter the VFT change ratio (0.95 ± 0.11) but have been shown to block the lowering effect of noradrenaline on the VFT (Murnaghan, 1975).

When the degree of O_2 lack was made less extreme (hypoxia) by gassing the McEwen's solution with a mixture of 5% CO_2 in air instead of pure N_2, the VFT was also reduced. The VFT change ratio of 0.75 ± 0.07 in 15 trials on 5 rabbit hearts was significantly different from unity ($P < 0.01$). Although the response with hypoxia was less marked than with anoxia they were not significantly different.

Since the introduction of isoprenaline inhalation for the treatment of bronchial asthma a number of fatalities have resulted. It has been conjectured that they may occur because the concomitant association of β-receptor cardiac stimulation and hypoxia induces ventricular fibrillation (McDevitt, Shanks & Swanton, 1974).

Since both isoprenaline (Murnaghan, 1975) and hypoxia cause a moderate fall in the VFT, the possibility had to be considered that in combination they might act synergistically which could account for the fatalities in the treatment of bronchial asthma. Consequently isoprenaline (4 nmol/min) was infused into two rabbit isolated hearts during perfusion with McEwen's solution gassed with 5% CO_2 in air. The fall of the VFT as indicated by its change ratio was not significantly greater during the combination of isoprenaline with hypoxia (0.60 ± 0.09 for 5 trials) than that with hypoxia alone (0.81 ± 0.11 for 2 trials). Furthermore, the effect of infusing isoprenaline alone (0.59 ± 0.04 for 3 trials) in the same two hearts was not significantly different from that when it was combined with hypoxia.

Duration and type of arrhythmia

Any procedure which converts a non-persistent to a persistent arrhythmia will predispose to a fatal outcome in the whole animal. Consequently the proportion of arrhythmias which were persistent in the perfused rabbit heart during the control and test periods have been compared. A similar comparison has been made in regard to whether the arrhythmia induced was a tachycardia or a fibrillation.

Anoxia significantly ($P < 0.001$) increased the incidence of a persistent arrhythmia (37 out of 40) compared with that of the corresponding controls (126/271) in the same 14 rabbit hearts. Adenosine alone (17/20, controls 30/61) had a similar effect in 3 rabbit hearts ($P < 0.02$). Dipyridamole alone (19/24; controls 59/111) also had a similar effect in 6 rabbit hearts ($P < 0.05$). All other possible comparisons were insignificant. No treatment

Table 2 Vulnerable times, mean ± s.e. mean ms, during control and test periods

	No. of hearts	*Control*	*Test*	P<
Anoxia	12	78.0 ± 4.2(196)	54.6 ± 4.2(23)	0.05
Anoxia + dipyridamole	6	75.5 ± 1.7(101)	57.5 ± 5.2(14)	0.01
Adenosine	3	78.8 ± 2.1 (59)	67.2 ± 3.2(18)	0.01
Anoxia + adenosine	1	87.9 ± 6.4 ·(14)	53.3 ± 13.3 (3)	0.01
β-Blockade	2	72.4 ± 4.7 (19)	72.9 ± 7.5(14)	NS
Anoxia + β-blockade	2		68.8 ± 3.9 (8)	NS

Number of values in parentheses.

significantly altered from the control the type of arrhythmia (42% fibrillations) which could be induced.

Vulnerable time

Since anoxia shortens the duration of the ventricular action potential one would expect that the vulnerable period would also be shortened. Because of variations among hearts, comparisons of the vulnerable times were restricted to those carried out on the same hearts. The results in Table 2 show that the vulnerable time was significantly shortened by anoxia alone or when combined with dipyridamole or adenosine and also by adenosine alone despite the fact that the heart rate was slowed in all cases. Dipyridamole alone or when combined with adenosine did not significantly alter the vulnerable time. However, β-adrenoceptor blockade prevented anoxia from significantly shortening the vulnerable time.

Discussion

The results of this study clearly indicate that a complete lack of O_2 (anoxia) in the perfusion fluid significantly lowers the ventricular fibrillation threshold (VFT) in the rabbit heart and increases the incidence of a persistent arrhythmia. Although a reduced amount of O_2 (hypoxia) in the perfusion fluid significantly lowered the VFT, the increase in the incidence of a persistent arrhythmia was small. These results on the duration of the arrhythmia in the isolated rabbit heart are in general agreement with those of Burn & Huković (1967) and suggest that a boundary or trigger zone between normal and anoxic myocardium which occurs with coronary occlusion (Harris, 1948) is not essential for sensitization of the ventricles to electrically-induced fibrillation. However, it would appear that this boundary zone may be of importance in order that ventricular fibrillation may occur spontaneously because the incidence of its occurrence is markedly reduced when the degree of anoxia of the infarcted area is reduced by the administration of hyperbaric oxygen (Smith & Lawson, 1962; Chardack *et al.*, 1964) and spontaneously arising ventricular fibrillation was not seen during O_2 lack in the rabbit isolated perfused heart. Such a boundary zone between normal and altered myocardium not only occurs in cardiac infarction but also where cold, drugs etc., are applied to a localized area of the myocardium.

The most important factor for the initiation of ventricular fibrillation is an asymmetry in recovery of excitability. This occurs normally during the repolarization phase of the myocardial action potential and only when a stimulus is applied during this vulnerable period is it capable of inducing ventricular fibrillation. During anoxia of the myocardium the duration of the action potential is reduced (Trautwein, Gottstein & Dudel, 1954). As the ventricles do not consist of a homogeneous population of fibres, asymmetry in excitability during late systole is likely to be exaggerated by anoxia as has been shown to occur with ischaemia (Han, 1964) and sympathetic activity (Han, de Jalon & Moe, 1964). Furthermore, differences in the degree of perfusion of various parts of the heart will magnify the asymmetry. Such lack of homogeneity in recovery of excitability will predispose to re-entry rhythms arising spontaneously or induced by a degree of stimulation less than normally required during the vulnerable recovery period. Assymmetry in the degree of depression of conduction by anoxia will contribute towards the initiation and maintenance of such re-entry rhythms. However, if maintenance of the anoxia is prolonged an excessive decrease in the membrane potential will reduce the excitability and so the likelihood of initiating an arrhythmia.

Some workers have produced evidence which indicates that the release of adenosine is responsible for the coronary vasodilatation produced by anoxia of the heart muscle. This is partly based on the fact that the vasodilator action of adenosine is potentiated by dipyridamole (Afonso & O'Brien, 1967) and lidoflazine (Afonso, O'Brien & Crumpton, 1968) which prevent the destruction and binding by the tissues of the released adenosine (Bunag, Douglas, Imai & Berne, 1964; Kolassa, Pfleger & Rummel, 1970; Afonzo & O'Brien, 1971). Three methods have been used to produce myocardial anoxia in the anaesthetized animal: coronary artery occlusion (ischaemia), asphyxia and systemic hypoxaemia. During coronary artery occlusion the adenosine content of the heart muscle is increased (Olsson, 1970), the adenosine is released into the coronary venous sinus (Rubio *et al.*, 1969) and the reactive hyperaemia after removal of the occlusion is potentiated by dipyridamole (Parratt & Wadsworth, 1972) and lidoflazine (Jageneau, Schaper & van Gerven, 1969). During asphyxia the amount of adenosine released into the pericardial sac has been shown to be increased (Rubio & Berne, 1969). However, the coronary vasodilating action of systemic hypoxaemia is not potentiated by dipyridamole (Parratt & Wadsworth, 1972) or lidoflazine (Afonso, 1969). Apparently only in the the first two is there evidence that adenosine may play a role in the induced vasodilatation. In the isolated heart, however, anoxia induced by perfusion with Ringer solution gassed with N_2

causes the release of adenosine and this effect is potentiated by dipyridamole (Berne, 1966). The effect of anoxia on ventricular irritability has been studied by noting the incidence of spontaneously arising ventricular fibrillation and by measuring the fall in the VFT in the anaesthetized animal. Coronary artery occlusion lowers the VFT in the infarcted area (Wiggers *et al.*, 1940) and gives rise to spontaneous ventricular fibrillation in a high proportion of trials (Badeer & Howarth, 1959). During asphyxia spontaneous ventricular fibrillation occurs in about one-third the number of trials (Coffman & Gregg, 1960). On the other hand systemic hypoxaemia neither predisposes to spontaneous ventricular fibrillation (Badeer & Howarth, 1959) nor does it lower the VFT (Rogers *et al.*, 1973). As indicated by this study, lack of O_2 induced by gassing the McEwen's solution with 5% CO_2 in N_2 or air lowers the VFT in the rabbit isolated perfused heart. It would appear therefore that anoxia in this preparation resembles more closely in its effects coronary artery occlusion or induced asphyxia rather than the induction of systemic hypoxaemia in the whole animal.

It is of interest to note that only in those cases where there is evidence that adenosine is released, did anoxia also either lower the VFT and/or predispose to spontaneous ventricular fibrillation. However, adenosine *per se* apparently is neither responsible for the fall in the VFT nor does it prevent the fall induced by anoxia in the rabbit isolated perfused heart. That it appears to play no role in this regard is substantiated by the results with dipyridamole which either alone or when combined with adenosine did not cause a fall in the VFT nor did it prevent anoxia from doing so. However, adenosine may play a permissive role in the maintenance of an induced arrhythmia because it significantly shortened the vulnerable time. As adenosine did not significantly alter the duration of the Q-T interval, the shortened vulnerable time suggests that vulnerability to a ventricular arrhythmia occurred earlier during repolarization. In the whole animal dipyridamole by preventing destruction and tissue binding of the released adenosine and thereby increasing coronary flow will reduce the effect of myocardial anoxia due to coronary occlusion or asphyxia and so offer protection against a fatal arrhythmia; its adenosine-sparing action consequently may act indirectly via coronary perfusion of collateral vessels rather than directly on the ventricular irritability. Although dipyridamole when combined with adenosine shortened the Q-T interval it did not alter the vulnerable time and it prevented adenosine from doing so. Consequently it appears to delay vulnerability until a later stage in the repolarization process which may have an antifibrillatory effect.

The fact that coronary artery occlusion and asphyxia predispose to ventricular fibrillation and cause the release of adenosine, while hypoxaemia does not, suggests that the former are probably more injurious procedures and produce actual damage to the tissue, either localized or widespread. The release of adenosine consequently may be an indicator of the degree of damage to the tissue which would be the determining factor predisposing to ventricular irritability. The isolated perfused anoxic heart would also fall under this category. Furthermore, additional factors may play a significant role in the initiation of an arrhythmia in the whole animal, e.g. release of catecholamines, localized increased concentration of extracellular potassium, pH decrease etc. It is well established that asphyxia is a potent stimulus to the sympathetic system and cardiac infarction in the dog has been shown to release catecholamines from the adrenal medulla (Ceremuzynski, Staszewska-Barczak & Herbaczynska-Cedro, 1969). The effect of the released catecholamines in the whole animal, however, are complex as Han *et al* (1964) have shown that infused adrenaline first lowers and then raises the VFT in the dog. Although anoxia causes the release of noradrenaline in the rabbit isolated perfused heart (Wollenberger & Shahab, 1965) and noradrenaline lowers the VFT (Murnaghan, 1975), the released noradrenaline cannot be responsible for the fall in the VFT induced by anoxia because the effect is not prevented by blockade of the β-adrenoceptors by propranolol or pindolol or by previous depletion of noradrenaline in the heart by reserpine.

McDevitt *et al* (1974) have shown that the sudden death induced by low doses of isoprenaline in hypoxaemic dogs is associated with cardiac standstill and not ventricular fibrillation. Clearly hypoxaemia does not sensitize the dog's heart to arrhythmic doses of isoprenaline. The fact that hypoxia in the rabbit isolated heart does not potentiate the lowering effect of isoprenaline on the VFT confirms that this combination does not predispose to ventricular arrhythmias. These findings suggest that sudden death in asthmatic patients under treatment with pressurized aerosols could be due to concomitant hypoxaemia if the cause of death is due to cardiac standstill but probably not if it is due to ventricular fibrillation.

The author wishes to thank the Irish Heart Foundation for financial support for this work. The material for this paper was presented in a preliminary form at the Fifth International Congress on Pharmacology in San Francisco, July 1972.

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(Received March 20, 1975.
Revised April 27, 1975.)

Br. J. Pharmac. (1975), 54, 421-427

THE EFFECTS OF HYPERCHOLESTEROLAEMIC PLASMA ON VASCULAR SENSITIVITY TO NORADRENALINE

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1 The pressor responses to injected noradrenaline (NA) of 42 isolated perfused femoral arteries of the rabbit were studied.

2 Potentiation of the responses was found when hypercholesterolaemic plasma was perfused through the arteries. No change was found with normal plasma.

3 Potentiation of the responses was found when isolated β-lipoprotein in Krebs solution was perfused. No change was found with similar amounts of bovine albumen.

4 Pure cholesterol dissolved directly into normal plasma, and dissolved via propanol into Krebs solution or plasma caused no potentiation. Propanol alone in Krebs or plasma had no effect.

5 Potentiation was caused by a decreased equilibrium coefficient (K_{eq}) for the NA-adrenoceptor interaction and an increased maximal pressor response (R_{max}).

6 It is concluded that cholesterol carried on its apoprotein is capable of potentiating the pressor effects of noradrenaline.

Introduction

It has been demonstrated that many steroids are capable of inhibiting the extraneuronal uptake of noradrenaline (NA) and related compounds in heart muscle (Iversen & Salt, 1970). Subsequently Salt & Iversen (1972) have demonstrated that cholesterol (when dissolved in alcohol and added to saline) is capable of a similar type of inhibition. Although the sensitivity of the heart muscle to NA was not studied, inhibition of the uptake processes would be expected to potentiate the effects of the hormone.

If the effects of cholesterol on vascular smooth muscle are similar to those in heart muscle an elevation of blood cholesterol levels should produce an increased vascular response to sympathetic activity. Burstyn, Horrobin & Miurin (1972) have shown that intravenous infusion of a sonicated cholesterol solution causes an increase in arterial blood pressure. Also, we have previously demonstrated that in obstructive jaundice where the serum cholesterol levels are elevated (Sherlock, 1968), there is a potentiated pressor response to injected noradrenaline (Bloom, Bomzon, Rosendorff & Scriven, 1974; Bloom, McCalden & Rosendorff, 1974). This circumstantial evidence would suggest that cholesterol is capable of causing a potentiated vascular response to noradrenaline.

Cholesterol is mainly carried in plasma on the β-lipoprotein and elevated levels of this protein are found in Fredrickson's type IIA hyperlipidaemia (Fredrickson, Levy & Lees, 1967).

We have, therefore, investigated the effects on the pressor response to NA of cholesterol in Krebs solution, cholesterol in hyperlipidaemic plasma and a β-lipoprotein extract. We have also attempted to dissolve cholesterol directly in normal plasma since sonication or dissolution in ethanol is unphysiological.

Methods

The arterial model used to assay the vasoconstrictor effect of NA was similar to that described by de la Lande & Rand (1965). The technique involved the measurement of perfusion pressure in a constant flow system.

New Zealand White rabbits were anaesthetized with intravenous pentobarbitone sodium (Nembutal, Abbott). A length (2-2.5 cm) of femoral artery was exposed, cannulated proximally, removed from the animal, washed through with a Krebs solution containing heparin and then perfused at constant flow in an organ bath with Krebs solution at 37° C. The bath was maintained at this temperature with an outer thermostatically controlled water jacket. The Krebs solution contained (mM) NaCl 118.0, KCl 4.69, NaH_2PO_4 1.33, $NaHCO_3$ 25.0, glucose 5.56, $CaCl_2$ 2.52 and $MgCl_2$ 1.05 and all perfused solutions were maintained at pH 7.40 and constant $P\text{CO}_2$ and $P\text{O}_2$ by bubbling a gas containing 6% CO_2 and 94% O_2 through the perfusate reservoir. Perfusion pressure was monitored with a Statham P 23 BB transducer connected to the flow system upstream of the artery via a T tube, and the perfusion rate was kept constant throughout each experiment at 4-5 ml/minute. This gave a perfusion pressure of 5-10 mmHg in the system.

The arterial constrictor responses to NA were determined by injection of graded doses of NA in Krebs solution at 37°C just proximal to the preparation. The resultant changes in vessel calibre and resistance were monitored as changes in perfusion pressure and recorded on a Beckman dynograph.

The system was allowed to stabilize for at least 30 min during perfusion with Krebs solution. Then a series of responses to graded doses of NA was obtained. The perfusate was then changed to the test solution, allowed to stabilize for 30 min and a further series of responses to NA was obtained. The perfusate was again changed to Krebs solution and 30 min later another series of responses was obtained.

Hyperlipidaemic plasma

Blood (60 ml) was taken from each of 15 fasting patients attending a special clinic for hyperlipidaemia. The only therapy was a diet and cholestryamine. All patients had elevated serum cholesterol levels and in each case it was confirmed by both ultracentrifugation and electrophoresis that these patients were true type IIA hyperlipidaemics (Fredrickson *et al.*, 1967). The blood was centrifuged for 3 min at 2500 rev/min and the supernatant plasma removed. Plasma urea and electrolytes were measured in each sample. In each experiment three of the plasma samples were selected at random, pooled and analysed for total cholesterol, β-cholesterol and triglycerides. Six experiments with hypercholesterolaemic plasma (HC) were performed and five with plasma from normal patients as controls.

Cholesterol in Krebs solution

Cholesterol (B.D.H.) was dissolved in propanol to give a stock solution of 50 mg/ml. The aliquots of this stock were added to Krebs solution to make a final concentration of 10 μg/ml to 50 μg/ml. Higher concentrations were not used because the cholesterol precipitated out of solution; five experiments were carried out. Three controls with propanol alone were also carried out.

β-Lipoproteins

Normal human plasma was spun in a Beckman C-265B ultracentrifuge at 19,000 rev/min for a period of 30 min to remove the chylomicrons. Then the samples were spun at 40,000 rev/min for 18 h with a density solution containing 11.4 g NaCl, 0.1 g disodium edetate (EDTA) and 1 ml 0.1 N NaOH made up to 1 litre with distilled water and of specific gravity 1.006. Pre-β-lipoproteins moved from plasma into this solution and were removed. The remaining plasma was further spun at 40,000 rev/min for 20 h with a second density solution containing 24.98 g NaBr in 100 ml of the first density solution and of specific gravity 1.182. The β-lipoproteins moved from plasma into this solution and their presence was confirmed with a Technicon AII autoanalyser as well as by electrophoresis. The β-lipoproteins were then dialysed against 0.9% w/v NaCl solution (saline) for 24 h to remove the EDTA and bromide. By this procedure the β-lipoprotein content in 600 ml of plasma was concentrated into 200 ml. This concentrate was then added to an equal volume of Krebs solution to give final concentrations of β-cholesterol 0.75 to 1.1 mg/ml. Five experiments were carried out in which these solutions were infused into femoral artery preparations, and an equal number of controls with bovine albumen (Merck) at 0.5 to 2.0 mg/ml.

Cholesterol in plasma

Cholesterol was added to normal human plasma either directly or from the propanol stock solution previously described. A maximum of 10 μg/ml of the cholesterol could be dissolved directly and 30 μg/ml with the propanol stock. Ten such experiments were carried out. Three controls with propanol alone in plasma were also performed.

Analysis of results

Two methods of analysing these results were used.

Method 1 The dose-response data obtained in each experiment were plotted as the increase in perfusion pressure (Response mmHg) against the logarithm of the dose (μg NA). This produced three curves for each experiment, one for control (Krebs) perfusate, one for the experimental perfusate and one for the washout with Krebs solution. The responses obtained when the plasma was perfused was compared to those of the first Krebs perfusion by a paired t test.

Method 2 The dose-response data obtained in each individual experiment were plotted as the reciprocal of the perfusion pressure increase (1/Response, mmHg) against the reciprocal of the dose (1/dose of NA, µg). This produced a straight line relation in each experiment as predicted from Michaelis-Menten kinetics for drug-receptor interaction (Goldstein, Aronow & Kaplan, 1965).

$$\frac{1}{R}=\frac{K_{eq}}{R_{max}}\cdot\frac{1}{D}+\frac{1}{R_{max}}$$

where R = response (mmHg), D = NA dose (µg), R_{max} = maximal response (mmHg), K_{eq} = equilibrium coefficient for drug-receptor interaction.

In each experiment values of R_{max} and K_{eq} were calculated from the intercepts of the line on the x and y axes respectively. When $1/D$ is zero, $1/R$ (y intercept) is equal to $1/R_{max}$. When $1/R$ is zero, $1/D$ (x intercept) is equal to $-1/K_{eq}$. Changes in $1/R_{max}$ imply changes in the intrinsic activity of the drug-receptor complex, while changes in $-1/K_{eq}$ imply changes in drug-receptor affinity.

Results

Hyperlipidaemic plasma (HC plasma)

Figure 1 shows three sets of responses obtained from a femoral artery. The top trace shows the pressure changes in the perfusate after injection of doses of NA ranging from 0.8 µg to 8.0 µg. The middle trace shows the pressure changes after injections of doses of NA ranging from 1.6 µg to 8.0 µg when the artery was perfused with HC plasma and the bottom trace after a period of washout with Krebs solution. It is clear that the responses to NA are much larger in the presence of the HC plasma. Table 1 shows the individual responses obtained in each of the six experiments before and during perfusion with HC plasma. Using a paired t test the responses with HC plasma were found to be significantly different from the first responses in Krebs solution ($P < 0.01$), in all of the six experiments. When normal plasma was perfused no potentiation was seen.

Another similar experiment is shown in Figure 2 as a log-dose *vs* response plot. The potentiation of the NA response in the presence of HC plasma is seen as a shift of the curve to the left of the control curve. After washout with Krebs solution the dose-responses were shifted back towards (but never quite reached) the initial curve.

Figure 3 shows a double reciprocal plot of the data from a typical experiment. In all of the experiments a high correlation coefficient was obtained (0.78–0.98). It can be seen that both the slope and the x intercept obtained with Krebs solution are different from those obtained during perfusion with HC plasma, whereas the y intercept is not significantly changed. Thus, the maximum response (R_{max}) of this artery has remained almost unchanged while there was a large change in the drug-receptor equilibrium coefficient (K_{eq}) (Goldstein *et al.*, 1968). Table 2 shows the values of K_{eq} and R_{max} for all HC experiments. Using a paired t test the K_{eq} (HC) was found to be significantly different ($P < 0.05$) from the K_{eq} (Krebs). Although there is a tendency for the R_{max} to be increased with the HC plasma this was not significant at the 5% level. There was no significant change in K_{eq} or R_{max} in the normal plasma controls. The assayed values of total cholesterol and % β-cholesterol are also shown in Table 2. There is no correlation between the degrees of NA potentiation and the total cholesterol concentrations.

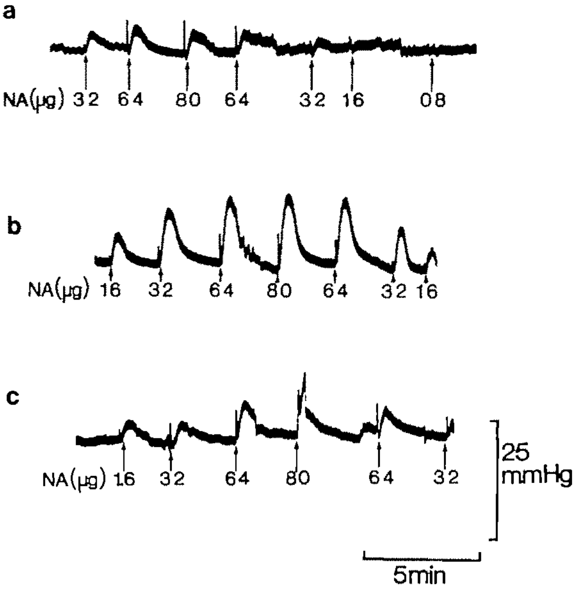

Figure 1 Three traces of perfusion pressure in isolated femoral arteries of the rabbit are shown. The top trace (a) shows the responses as changes in perfusion pressure (mmHg) when several doses of noradrenaline (NA) were injected during perfusion with Krebs solution. Trace (b) shows similar responses when hypercholesterolaemic plasma (HC plasma) was perfused and trace (c) those obtained during reperfusion with Krebs solution. A time scale (min) and a pressure calibation are also shown. HC plasma potentiates the effects of NA.

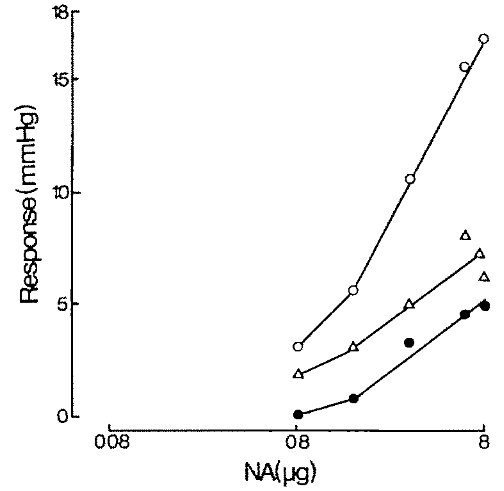

Figure 2 The pressure responses (mmHg) on the y axis are plotted against the log of the dose of noradrenaline (NA, µg) on the x axis. The responses obtained during hypercholesterolaemic plasma perfusion (○) are shifted to the left of those during initial Krebs solution perfusion (●). Washing with Krebs solution (△) caused partial reversal of this effect.

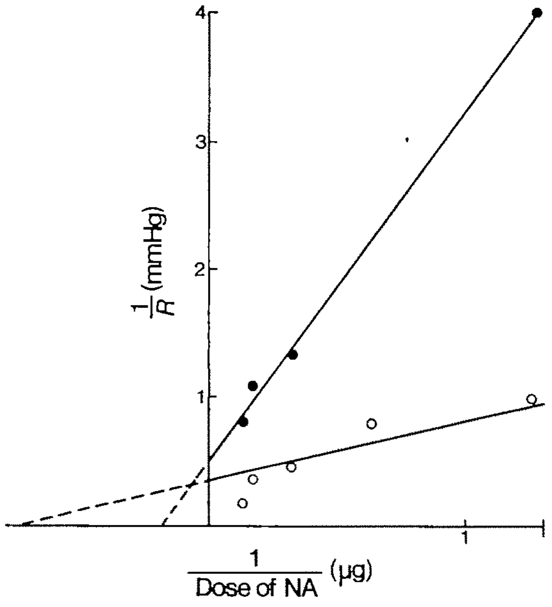

Figure 3 The reciprocal of response (mmHg) on the y axis is plotted against the reciprocal of the dose of noradrenaline (NA, µg) on the x axis. The slope and the x intercept obtained during hypercholesterolaemic plasma perfusion (○) are different from those obtained during Krebs solution perfusion (●). There is no difference in the y intercepts. Thus the K_{eq} value has changed but the tissues maximal response (R_{max}) has not.

Cholesterol in Krebs solution

In the five experiments carried out no potentiation of the response to noradrenaline was found at any of the concentrations (10 µg/ml to 50 µg/ml) used.

Table 1 The perfusate pressure changes (mmHg) in six experiments when noradrenaline was injected in doses ranging from 0.8 to 8.0 µg. (The values are shown during Krebs (K) and during hypercholesterolaemic plasma (HC) perfusion)

		NA dose (µg)					
Expt. no.		*0.8*	*1.6*	*3.2*	*6.4*	*8.0*	
1	K	0	1.2	3.4	4.7	5.0	$P < 0.01$
	HC	2.5	5.3	10.6	14.7	17.5	
2	K	0	0.5	0.7	1.3	1.5	$P < 0.01$
	HC	1.0	1.7	3.8	5.0	5.0	
3	K	1.8	2.9	3.7	6.6	8.8	$P < 0.01$
	HC	5.1	10.3	16.9	19.8	22.1	
4	K	0.2	1.0	0.7	0.9	1.2	$P < 0.01$
	HC	1.1	1.3	2.1	2.7	6.2	
5	K	6.3	7.5	16.9	30.0	–	$P < 0.01$
	HC	15.0	33.7	43.7	–	–	
6	K	0.9	2.5	4.7	6.9	6.6	$P < 0.025$
	HC	8.4	13.8	18.8	–	–	

The results of paired t tests are shown on the right of the table.

β-Lipoproteins

Figure 4 shows the responses obtained to NA in an isolated artery before, during and after perfusion with 1 mg/ml of β-lipoproteins. Although the doses of NA used with lipoprotein were one tenth of those in Krebs solution, much larger responses were obtained. This would indicate a shift of the log dose-response curve to the left during protein perfusion. Comparison by a paired *t* test of the responses in Krebs solution to the responses when β-lipoprotein was added, showed a significant difference in all five experiments ($P < 0.01$).

Table 3 shows the calculated values of K_{eq} and R_{max} (from a Lineweaver-Burke plot) for all five experiments with β-lipoprotein. A decrease in K_{eq} and an increase in R_{max} occurred. No change in sensitivity to NA occurred in any of the controls when bovine albumen was used.

Cholesterol in plasma

The maximum possible concentration of cholesterol produced no potentiation of the response to NA in any of the 10 experiments carried out. We found that propanol added either to Krebs solution or to normal plasma had no effect on the vascular response to NA.

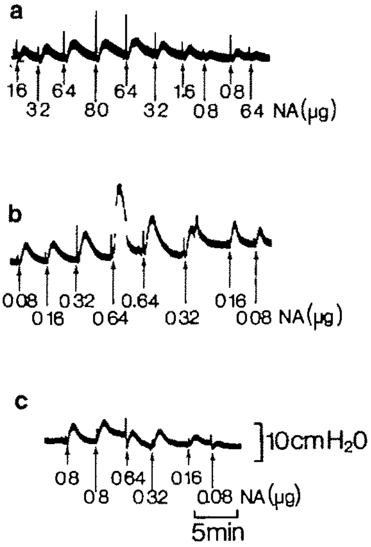

Figure 4 The responses to noradrenaline (NA) obtained in one experiment are shown (a) during Krebs solution perfusion; (b) during β-lipoprotein perfusion (1 mg/ml), and (c) after a washout of the β-lipoprotein with Krebs solution. Time and pressure calibrations are shown. It can be seen that the lipoprotein potentiates the response to NA.

Table 2 The equilibrium coefficient (K_{eq}) and the maximal possible response (R_{max}) calculated for each hypercholesterolaemic plasma experiment and each normal plasma control

		Hypercholesterolaemia experiments				
Expt. no.	*Total cholesterol mg/100 ml*	*β-cholesterol %*	K_{eq} *Krebs*	K_{eq} *HC Plasma*	R_{max} *Krebs*	R_{max} *HC Plasma*
1	525	75	37.1	27.7	30.3	90.9
2	415	72	6.6	8.7	2.5	11.9
3	370	70	3.4	5.5	9.4	41.7
4	326	71	5.3	2.4	1.9	4.1
5	490	70	4.0	0.1	34.5	4.6
6	300	73	7.4	2.3	14.3	32.3
			$P < 0.05$		NS	
		Normal plasma controls				
1	170	65	15.0	2.9	12.5	1.5
2	165	69	8.9	2.9	33.3	16.6
3	200	70	1.1	2.2	4.3	5.3
4	195	65	2.7	8.0	25.0	27.7
5	201	68	2.9	4.2	6.7	9.1
			NS		NS	

The significance (paired *t* test) of any changes are shown at the base of each column. Also shown are the values of total cholesterol (mg%) and the % β-cholesterol for each of the plasmas used.

Table 3 The values of the equilibrium coefficient (K_{eq}) for the noradrenaline (NA)-receptor interaction and the maximal response (R_{max}) to NA in the five experiments when Krebs solution was followed by perfusion with a β-lipoprotein solution.

	K_{eq}		R_{max} (cmH_2O)	
Expt. No.	*Krebs*	*β-protein*	*Krebs*	*β-protein*
1	0.85	0.19	5.08	16.95
2	5.14	5.86	22.73	71.43
3	2.49	0.75	11.49	15.53
4	2.42	1.44	13.89	55.56
5	13.89	2.23	52.63	58.82
Mean	4.96	2.09	21.16	43.66

Discussion

When pure cholesterol was either dissolved in plasma or in Krebs solution no potentiation of the effects of noradrenaline (NA) were observed. The concentrations used were in excess of those used by Salt & Iversen (1972) in heart muscle but lower than those found pathologically. Salt & Iversen reported that the extraneuronal uptake of NA into heart muscle was inhibited. Our failure to demonstrate potentiation of the responses to NA in the vascular preparation may mean that the vascular muscle is not as sensitive to cholesterol as heart muscle and that much larger concentrations of cholesterol are needed.

Large increments of plasma cholesterol are found in Fredrickson's type IIA hyperlipidaemia (Fredrickson *et al.*, 1967). This is largely carried in plasma as an increase in the β-lipoprotein fraction. When plasma from these patients was used, potentiation of the responses to NA was obtained. This potentiation might have been achieved either because the concentration of cholesterol was much higher than in the previous experiments; or because when cholesterol is attached to its apoprotein it is much more effective. There may also have been some other factor in the hyperlipidaemic plasma which potentiated the response to NA. However, this seems unlikely as our experiments with isolated β-cholesterol (attached to β-lipoprotein) would indicate that this constituent of the plasma was primarily responsible for the potentiation. This would strengthen the hypothesis that cholesterol attached to its apoprotein is more effective than pure dissolved cholesterol.

The analysis of the data by Michaelis-Menten kinetics (Goldstein *et al.*, 1968) indicates that when the vascular muscle is exposed to a high cholesterol concentration there is a decreased equilibrium coefficient (K_{eq}) for the NA-adrenoceptor interaction and an increased maximal response (R_{max}). The decrease in K_{eq} would imply that more NA-adrenoceptor complex was formed for a given dose of NA. Thus, either the affinity of the receptors for NA was increased or more of the injected NA became available for combination at the receptor site. An inhibition of extraneuronal uptake of NA into the vascular muscle would result in more NA becoming available for receptor combination. In view of the work of Salt & Iversen (1972) this mechanism seems the most likely. However, we cannot rule out some increase in receptor affinity. The overall results of either mechanism is a move to the right in the equation NA + adrenoceptor $\rightleftharpoons$ NA/adrenoceptor complex.

The increase in R_{max} was found to be significant only with pure β-lipoprotein. However, there was an upwards trend with the HC plasma. This increase must be associated with an increased intrinsic activity of the tissue and may be a result of tissue hyperexcitability or the recruitment of additional adrenoceptors. We consider either of these explanations possible.

These results would, therefore, indicate that when vascular muscle is exposed to cholesterol at a high concentration the muscle becomes more sensitive to NA. This hypersensitivity may be mediated (at least in part) by an inhibition of extraneuronal uptake mechanisms, similar to that previously reported in heart muscle.

We would like to thank Professor Mendelsohn, Dr E. Stein and his staff for collection of the plasma and cholesterol estimations. Also, Dr C. Altbrecht of the Council for Scientific and Industrial Research for assistance with the lipoprotein preparation. Reprint requests to T.A. McC.

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(Received March 31, 1975.)

Br. J. Pharmac. (1975), 54, 429-436

INFLUENCE OF ANTI-HYPERTENSIVE DRUG TREATMENT ON VASCULAR REACTIVITY IN SPONTANEOUSLY HYPERTENSIVE RATS

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1 The effect of prolonged anti-hypertensive drug treatment on the blood pressure of conscious spontaneously hypertensive rats (SH-rats), and of age-matched normotensive Sprague-Dawley rats was determined during the development of hypertension in SH-rats and in the early stages of established hypertension. A comparison of the vascular reactivity to noradrenaline (NA) and 5-hydroxytryptamine (5-HT) was also made in isolated perfused mesenteric artery preparations from treated and control SH- and Sprague-Dawley rats.

2 Chronic treatment from age 4 to 16 weeks with hydrallazine alone, or a combination of hydrallazine/hydrochlorothiazide/reserpine, *ad libitum* in the drinking water, prevented the development of hypertension in SH-rats and also reduced the vascular reactivity to NA and 5-HT in isolated vessel preparations from treated compared to control rats.

3 Similar drug treatments started in early established hypertension reduced blood pressure in SH-rats over the 12 week treatment period (from age 8 to 20 weeks) without affecting vascular reactivity to NA and 5-HT in the isolated vessel preparation.

4 Drug treatments had little effect on blood pressure of age-matched Sprague-Dawley rats and no effect on vascular reactivity to NA and 5-HT in the isolated perfused mesenteric artery preparation from treated compared to control rats.

5 These results indicate that the development of increased vascular reactivity and of hypertension in SH-rats occurs simultaneously and, therefore, the vascular changes may be a consequence of the structural changes induced by the raised blood pressure.

6 In established hypertension, no regression of vascular changes was observed despite prolonged reduction of blood pressure. The rôle of an increased vascular reactivity in the maintenance of hypertension is therefore questionable.

Introduction

Folkow, Grimby & Thulesius (1958) suggested that the raised peripheral vascular resistance in essential hypertension in man may be largely due to structural changes in the resistance vessels. The raised blood pressure may induce these adaptive structural changes so that an increased wall/lumen ratio develops and is responsible for the increased vascular reactivity to vasoconstrictor stimuli in hypertensive, compared to normotensive, states (Folkow, 1956; Folkow *et al.*, 1958; Folkow & Sivertsson, 1968; Folkow, Hallbäck, Lundgren & Weiss, 1970a; Hallbäck, Lundgren & Weiss, 1974).

Experimental data support these theoretical derivations and demonstrate that an increased vascular reactivity to vasoconstrictor stimuli exists in clinical hypertension (Conway, 1963; Sivertsson & Oleander, 1968) and in different types of experimental hypertension in rats whether in the whole animal preparation (Folkow *et al.*, 1970a) or in perfused hindquarters (Folkow, Hällback, Lundgren & Weiss, 1970b) or isolated perfused mesenteric artery preparation (Haeusler & Haefely, 1970; Haeusler & Finch, 1972). However, the work of others (Bandick & Sparks, 1970; Bohr & Sitrin, 1970; Harris, 1972; Holloway & Bohr, 1973) suggests that the adaptive structural changes lead to the individual smooth muscle cells becoming supersensitive to vasoconstrictor stimuli.

More recently it has been shown that interference with the development of hypertension in spontaneously hypertensive rats (SH-rats) by immunosympathectomy (Folkow, Hällback, Lundgren & Weiss, 1972), anti-hypertensive drugs (Weiss, 1974; Weiss, Lundgren & Folkow, 1974) or by the production of regional hypotension by aortic clipping (Folkow, Gurevich, Hällback, Lundgren & Weiss, 1971) alters the vascular activity of the perfused hindquarters to noradrenaline; in renal hypertensive rats, removal of the renal artery clip results in a fall in blood pressure and regression of vascular changes (Lundgren, 1974). However, in established hypertension, the work of Weiss (1974) and of Weiss *et al.* (1974) indicates that the regression of vascular changes is less likely and none was observed in deoxycorticosterone acetate (DOCA)/saline hypertensive rats after short-term anti-hypertensive treatment (Finch, 1974).

In the present study the relationship between the level of blood pressure in SH-rats and vascular reactivity to noradrenaline and 5-hydroxytryptamine in the isolated mesentery preparation has been investigated by preventing the development of hypertension in these rats by continuous anti-hypertensive drug therapy; in addition SH-rats were subjected to similar therapy in the early stages of established hypertension to determine whether the structural changes regressed after reduction of the elevated blood pressure to approximately normotensive levels for 12 weeks.

Methods

Age-matched female spontaneously hypertensive rats (SH-rats) (Japanese strain; Okamoto & Aoki, 1963) and female Sprague-Dawley rats were used for these studies.

Isolated perfused mesenteric artery preparation

For the determination of vascular reactivity to noradrenaline (NA) and 5-hydroxytryptamine (5-HT) in this preparation, rats were anaesthetized with ether and after cannulation of the superior mesenteric artery the mesentery was isolated as described by McGregor (1965).

The isolated preparation was floated on the surface of a 50 ml organ bath filled with Krebs-Henseleit solution at 37° C and bubbled with 95% O_2 and 5% CO_2 (Haeusler & Haefely, 1970). The preparation was perfused through the cannulated mesenteric artery with Krebs-Henseleit solution at a constant flow rate of 6-8 ml/min (to give a basal perfusion pressure of approximately 40 mmHg); perfusion pressure was recorded with a Statham P23Db pressure transducer.

Dose-response curves to NA and 5-HT were obtained by injecting these drugs into the perfusion system just anterior to the cannulated artery. Single injections (10 μl) were given at 2 min intervals and the maximum increase in perfusion pressure was recorded.

Indirect measurement of blood pressure

Before the *in vitro* experiments, and during the course of anti-hypertensive treatment described below, systolic blood pressure was measured in the conscious rat by the indirect tail cuff method, using the appropriate size of sensor and pressure cuff (W + W recorder); body weight was also recorded before the *in vitro* study.

Anti-hypertensive drug treatment

Preliminary studies showed that the blood pressure of female SH-rats rose from normotensive levels of 130 mmHg at an age of 5 weeks to 180 mmHg at 8 weeks of age and that from 9 to 20 weeks of age blood pressure was maintained at 200 mmHg.

Table 1 Effect of anti-hypertensive drug treatment on the development of hypertension in spontaneously hypertensive rats and on the blood pressure of age-matched normotensive Sprague-Dawley rats

Animals	*Treatments*	*Blood pressure (mean ± s.e. mean) (mmHg) at age (weeks)*		
		7	*13*	*16*
Spontaneously hypertensive rats	Water (n = 16)	180.7 ± 3.3	207.9 ± 3.8	196.6 ± 2.6
	Hydrallazine/reserpine/ hydrochlorthiazide (n = 7)	150 ± 2.7 ($P < 0.001$)	144.4 ± 1.9 ($P < 0.001$)	132.6 ± 4.6 ($P < 0.001$)
	Hydrallazine (n = 7)	152 ± 6 ($P < 0.001$)	162 ± 7.2 ($P < 0.001$)	138.6 ± 5.1 ($P < 0.001$)
Normotensive Sprague-Dawley rats	Water (n = 16)	127 ± 1.8	–	130 ± 1.8
	Hydrallazine/reserpine/ hydrochlorthiazide (n = 16)	130 ± 2.6 ($P > 0.05$)	–	110 ± 4.4 ($P < 0.001$)
	Hydrallazine (n = 8)	135 ± 3.5 ($P > 0.05$)	–	131.6 ± 3 ($P > 0.05$)

Treatment was started at age 4 weeks, for details of treatments see Methods section; n is the number of rats per group; P values indicate significant difference from water-treated control group.

Drug treatments were started at an age of 4 weeks, i.e. before the development of hypertension and at an age of 8 weeks, i.e. in the early stages of established hypertension.

Groups of SH-rats aged 4 weeks, received the following combination of anti-hypertensive drugs *ad libitum* in their drinking water: hydrallazine 80 mg/l, hydrochlorothiazide 100 mg/l, reserpine 1.0 mg/l or hydrallazine 80 mg/l alone. Treatment was continued for 12 weeks (to 16 weeks of age); control groups received water alone. Age-matched Sprague-Dawley rats were subjected to the same treatment or received water alone. Groups of SH-rats and Sprague-Dawley rats, aged 8 weeks, received the same treatments as the younger rats for a period of 12 weeks (to 20 weeks of age).

Drugs used were hydrallazine hydrochloride (Ciba), hydrochlorothiazide (MSD), 5-hydroxytryptamine creatinine sulphate (5-HT), noradrenaline bitartrate (NA) and reserpine (Koch-Light). Doses of hydrochlorothiazide, 5-HT and NA are expressed as base.

Results

Effect of anti-hypertensive drug therapy on the development of hypertension in SH-rats

Table 1 shows that each drug treatment effectively prevented the development of hypertension in SH-rats whilst blood pressure rose over the same 12 week period in SH-rats drinking water alone. Three weeks after treatment started (7 weeks of age) the blood pressure of each treated group was in the region of 150 mmHg compared to a value of 180 mmHg in the untreated control group; after 12 weeks treatment (16 weeks of age) the blood pressure of the hydrallazine/hydrochlorothiazide/reserpine group was 60 mmHg lower than that of the control group whilst that of the hydrallazine alone group was 60 mmHg lower than that of the untreated SH-rats.

Apart from a significant reduction, at 16 weeks of age, of 20 mmHg in the hydrallazine/hydrochlorothiazide/reserpine group, the blood pressure of Sprague-Dawley rats subjected to drug therapy did not differ from that of Sprague-Dawley rats drinking water alone.

Effect of anti-hypertensive drug therapy on the blood pressure of SH-rats with established hypertension

Throughout the 12 week treatment period each drug treatment significantly lowered the blood pressure of SH-rats compared with that of untreated SH-rats (Table 2). After 12 weeks treatment (age 20 weeks) the blood pressure of the hydrallazine/hydrochlorothiazide/reserpine group was approximately 100 mmHg lower than that of control rats drinking water whereas that of the hydrallazine group was approximately 90 mmHg lower than that of the control rats.

After 12 weeks of similar treatments the blood pressure of treated normotensive Sprague-Dawley rats was not significantly different from that of control Sprague-Dawley rats (Table 2).

Table 2 Effect of anti-hypertensive drug treatment on the blood pressure of spontaneously hypertensive rats in the early stages of established hypertension and on the blood pressure of age-matched normotensive Sprague-Dawley rats

Animals	*Treatments*	*Blood pressure (mean ± s.e. mean) (mmHg) at age (weeks)*			
		7 (pre-dose)	*10*	*16*	*20*
Spontaneously hypertensive rats	Water ($n = 8$)	190 ± 5	197 ± 2.5	195 ± 5.2	209 ± 5
	Hydrallazine/reserpine/ hydrochlorthiazide ($n = 9$)	189 ± 4.6 ($P > 0.05$)	149 ± 3.4 ($P < 0.001$)	116 ± 3 ($P < 0.001$)	111 ± 4.3 ($P < 0.001$)
	Hydrallazine ($n = 8$)	—	151 ± 5 ($P < 0.001$)	128 ± 4.2 ($P < 0.001$)	129 ± 5.9 ($P < 0.001$)
Normotensive Sprague-Dawley rats	Water ($n = 8$)	—	127 ± 2.6	131 ± 3	125.7 ± 3.1
	Hydrallazine/reserpine/ hydrochlorthiazide ($n = 9$)	—	140 ± 4.3 ($P < 0.02$)	135 ± 7 ($P > 0.05$)	126 ± 7.6 ($P > 0.05$)
	Hydrallazine ($n = 8$)	—	137.3 ± 3.3 (($P < 0.02$)	133 ± 5 ($P > 0.05$)	128 ± 3.2 ($P > 0.05$)

Treatment was started at age 7 weeks; n is the number of rats per group; P values indicate significant difference from water-treated control group.

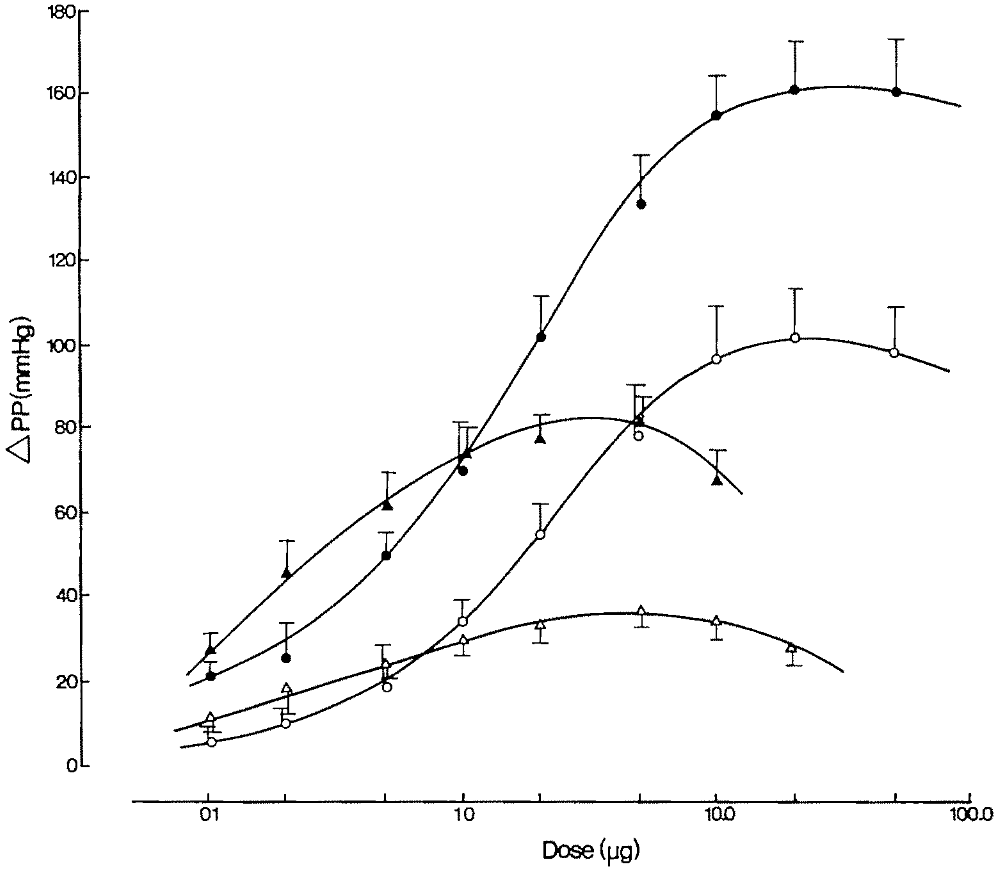

Figure 1 Dose-response curves for the increase in perfusion pressure (ΔPP) (mmHg) produced by noradrenaline (NA) and 5-hydroxytryptamine (5-HT) in isolated perfused mesenteric artery preparations from age-matched spontaneously hypertensive rats drinking water (controls) or treated with water containing hydrallazine/hydrochlorothiazide/reserpine, from age 4 to 16 weeks. From control rats, for NA responses (●) *n* = 8, BP = 206 ± 5.2 mmHg, body weight = 207 ± 3 g and for 5-HT responses, (▲) *n* = 8, BP = 196 ± 4.2 mmHg, body weight = 205.5 ± 3 g. From treated rats, for NA responses (○) *n* = 8, BP = 125 ± 8.2 mmHg, body weight = 196 ± 4.5 g and for 5-HT responses (△) *n* = 8, BP = 127 ± 7.5 mmHg, body weight = 191 ± .3 g. Mean values with s.e. mean are shown or quoted.

Vascular reactivity to noradrenaline and 5-hydroxytryptamine in isolated perfused mesentery preparation

Since Sprague-Dawley rats were used as controls, and not rats of the Wistar strain from which the Japanese strain of SH-rats were originally derived, no attempt is made to compare the vascular reactivity to NA and 5-HT of preparations from SH-rats with that of Sprague-Dawley rats. The Sprague-Dawley rats were considered to serve as adequate controls for the determination of any effect of the drug treatments *per se* on vascular reactivity to NA and 5-HT

Effect of prevention of development of hypertension in SH-rats on vascular reactivity

After treatment from age 4 to 16 weeks with a combination of hydrallazine/hydrochlorothiazide/reserpine, during which time the development of hypertension in SH-rats was prevented (Table 1), the vascular reactivity to NA and 5-HT of 16-week-old SH-rats was significantly reduced compared to that of water-treated SH-rats (Figure 1). However, the vascular reactivity to those amines of preparations from treated and control Sprague-Dawley rats did not differ (Figure 2). Similarly anti-hypertensive therapy by hydral-

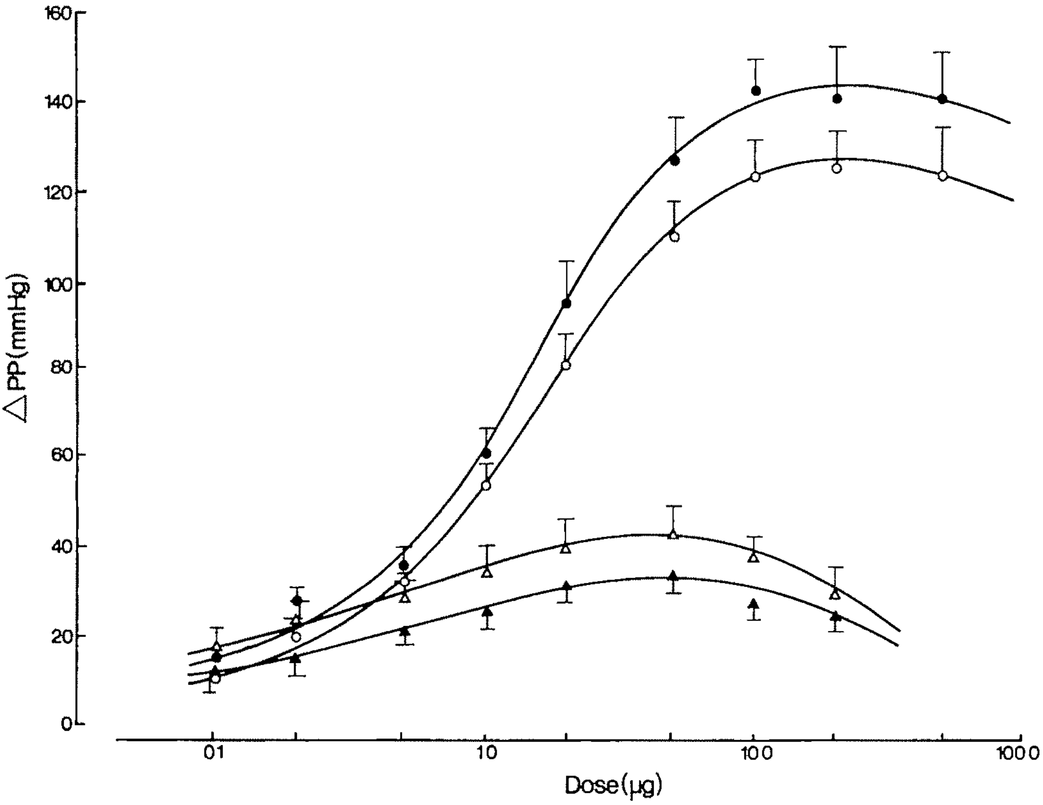

Figure 2 Dose-response curves for the increase in perfusion pressure (△PP) (mmHg) produced by noradrenaline (NA) and 5-hydroxytryptamine (5-HT) in isolated perfused mesenteric artery preparations from age-matched normotensive Sprague-Dawley rats drinking water (controls) or treated with water containing hydrallazine/hydrochlorothiazide/reserpine, from 4 to 16 weeks. From control rats, for NA responses (●) n =7, BP = 128 ± 3 mmHg, body weight = 235 ± 3.2 g and for 5-HT responses (▲) n = 7, BP = 129 ± 3 mmHg, body weight = 246 ± 5.5 g. From treated rats, for NA responses (△) n = 7, BP = 105 ± 4 mmHg, body weight = 233 ± 6.99 g and for 5-HT responses (△) n = 7, BP = 99 ± 3.4 mmHg, body weight = 228 ± 5 g. Mean values with s.e. mean are shown or quoted.

lazine alone reduced the vascular reactivity to NA and 5-HT of 16 week SH-rats but did not affect those of similarly treated Sprague-Dawley animals.

Effect of anti-hypertensive drug treatment on vascular reactivity in established hypertension

After treatment from age 8 to 20 weeks with hydrallazine/hydrochlorothiazide/reserpine the vascular reactivity to NA and 5-HT of 20-week-old SH-rats was not significantly different from that of water-treated controls (Figure 3). The vascular reactivity to NA and 5-HT of preparations from age-matched Sprague-Dawley rats did not differ from that of water-treated controls. Similarly, treatment with hydrallazine alone did not affect vascular reactivity to NA and 5-HT in preparations from either SH-rats or Sprague-Dawley rats.

Discussion

As shown by others (Okamoto & Aoki, 1963; Freis, Regan, Pillsbury & Matthews, 1972) the development of hypertension in spontaneously hypertensive rats occurred from birth until the animals were 3-4 months of age. This growth period is therefore critical for the development of raised blood pressure and for this reason chronic anti-hypertensive therapy intended to prevent the development of hypertension was started at 4 weeks of age; other workers (Freis *et al.*, 1972; Weiss *et al.*, 1974) considered that starting drug treatment at 10 weeks of age was adequate.

The hydrallazine/hydrochlorothiazide/reserpine drug combination used as anti-hypertensive therapy in the present investigation was found, as reported by Freis *et al.* (1972), to prevent the

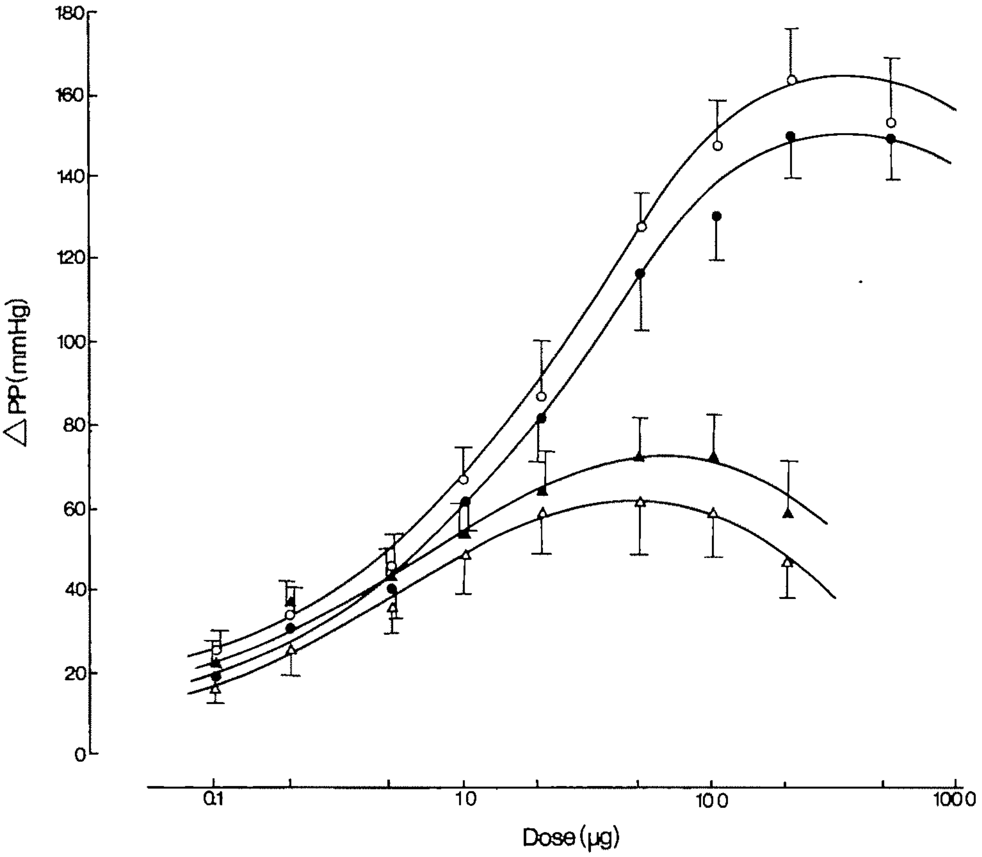

Figure 3 Dose-response curves for the increase in perfusion pressure (△PP) (mmHg) produced by noradrenaline (NA) and 5-hydroxytryptamine (5-HT) in isolated perfused mesenteric artery preparations from age-matched spontaneously hypertensive rats drinking water (controls) or treated with water containing hydrallazine/hydrochlorothiazide/reserpine), from age 8 to 20 weeks. From control rats, for NA responses (●) $n = 8$, BP = 209 ± 5.2 mmHg, body weight = 211 ± 3 g and for 5-HT responses (▲) $n = 8$, BP = 199 ± 2.3 mmHg, body weight = 222.6 ± 4 g. From treated rats, for NA responses (○) $n = 8$, BP = 110 ± 2.8 mmHg, body weight = 186 ± 12 g and for 5-HT responses (△) $n = 8$, BP = 101 ± 8 mmHg, body weight = 191 ± 4.5 g. Mean values with s.e. mean are shown or quoted.

development of hypertension in SH-rats; additionally, hydrallazine alone was also effective in a similar manner. In older SH-rats the administration of hydrallazine alone, or of the drug combination, reduced and maintained the blood pressure of SH-rats to levels approaching those of normotensive rats.

By contrast these drug treatments had little effect on the blood pressure of normotensive Sprague-Dawley rats and moreover these treatments themselves did not significantly alter the vascular reactivity to NA and 5-HT of isolated mesentery preparations from these rats.

The vascular reactivity to NA and 5-HT in preparations from SH-rats treated to prevent the development of hypertension was significantly reduced compared to that in untreated SH-rats. This effect complied with two of Folkow's criteria for reduced vascular reactivity, i.e. less steep dose-response curve and reduced maximum increase in perfusion pressure but threshold doses were less effective in treated SH-rats. However, commencement of similar treatments in SH-rats which were in the early stages of established hypertension failed to reduce vascular reactivity compared to that in untreated SH-rats. These results, therefore, indicate that the critical period for the development of vascular reactivity changes in this preparation is the same as that for the development of hypertension and moreover that after vascular changes have occurred regression is not obtained after a prolonged period of normalization of the blood pressure.

In DOCA/saline hypertensive rats, Finch (1974) found that similar drug treatment for 4 weeks whilst reducing blood pressure to normotensive levels failed to modify vascular reactivity to NA, 5-HT or adenosine 5′-triphosphate in the isolated mesentery preparation. However, in contrast to these findings, Weiss (1974) found that anti-hypertensive treatment with a hydralazine/guanethidine combination for up to 20 weeks reduced the blood pressure of 8-month-old SH-rats and also caused partial regression of vascular changes in the perfused hindquarters of treated rats. In the renal hypertensive rat Lundgren (1974) found that removal of the renal artery clip after hypertension had developed led to a rapid fall in blood pressure and regression of the increased vascular reactivity in the perfused hindquarters. Additionally, in 8-12-month old SH-rats, Weiss & Hallback (1974) reported that partial regression of vascular changes occurred in the hindquarters after regional hypotension was induced by aortic ligation for short periods of time. The different results obtained in rats with established hypertension in this study, from those of Folkow's group, is probably accounted for by the presence of intact blood vessels, including resistance vessels, in the hindquarters, but not the mesentery, preparation.

In contrast to results in established hypertension, prevention of the development of hypertension in SH-rats by immunosympathectomy (Folkow *et al.*, 1972) or by the chronic administration of propranolol (Weiss *et al.*, 1974) also prevented the raised vascular reactivity to NA that occurs in the perfused hindquarters of SH-rats. The present study has shown that anti-hypertensive drug treatment that prevents the development of hypertension in SH-rats also prevents the increased vascular reactivity to NA and 5-HT in isolated mesentery preparations. These results, therefore, support the hypothesis of Folkow and his co-workers that a raised transmural pressure is the main determinant of the structural changes in the wall/lumen ratio of blood vessels and represents a vascular adaptation to the raised perfusion pressure.

Moreover, since the vascular changes did not regress despite prolonged reduction in blood pressure the increased vascular reactivity is of doubtful significance in the maintenance of the elevated blood pressure. It remains possible that reduction in blood pressure for more prolonged periods will result in a reduced vascular reactivity.

I am indebted to Miss V. Chapman for skilled technical assistance and to Merck, Sharp and Dohme Ltd. and Ciba-Geigy Ltd. for generous donations of drugs.

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(Received February 18, 1975.
Revised April 28, 1975.)

Br. J. Pharmac. (1975), 54, 437-443

AN INCREASED REACTIVITY IN HYPERTENSIVE RATS UNAFFECTED BY PROLONGED ANTIHYPERTENSIVE THERAPY

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1 Isolated perfused mesenteric arteries obtained from chronic experimental hypertensive rats (deoxycorticosterone/NaCl) exhibited an increased reactivity to noradrenaline, 5-hydroxytryptamine and adenosine 5′-triphosphate (ATP) when compared with similar preparations from age-matched normotensive animals.

2 The dose-response curves to all three vasoconstrictor agents obtained from hypertensive animals exhibited a steeper slope, and higher maximum without any significant change in the threshold dose suggesting that adaptive/structural changes in the blood vessels had taken place.

3 Ten week treatments with antihypertensive combinations of hydrallazine, hydrochlorothiazide and reserpine or hydrallazine and mecamylamine lowered the systolic blood pressures of the hypertensive rats to those of normotensive animals and also reversed secondary changes such as periarteritis nodosa of the mesentery and cardiac hypertrophy.

4 The reactivity of these blood vessels to all these vasoconstrictor agents from the hypertensive rats with a normalized blood pressure was similar to those obtained with untreated hypertensive animals.

5 The persistent increased reactivity in the hypertensive rats after long-term anti-hypertensive treatment suggests that the hyperresponsiveness is secondary to the elevated blood pressures and that the adaptive/structural changes of the blood vessels in chronic hypertensive rats cannot be reversed by prolonged antihypertensive therapy.

Introduction

In several models of experimental hypertension a hyperreactivity has been reported for various vasoconstrictor stimuli (McGregor & Smirk, 1970; Finch, 1971, 1974a; Nicholas, 1971). Since this increased reactivity can be demonstrated in several isolated preparations it would seem to be independent of both circulating humoral agents and neurogenic control (Haeusler & Haefely, 1970; Haeusler & Finch, 1972; Beilin & Ziakas, 1972).

The underlying mechanism for this hyperresponsiveness and its role in hypertension is not clear. However, one possible mechanism is that due to the elevated blood pressure, adaptive structural changes take place in the blood vessels resulting in an increased wall/lumen ratio (Folkow, 1971; Weiss, 1974a; Lundgren, 1974a). As a consequence of the adaptive/structural changes, theoretical and experimental data show that the slope of dose-response curves to various vasoconstrictor stimuli become steeper and a higher maximum is obtained. Also, in most cases there is no change in the threshold dose for the various agonists (Folkow, Hallbäck, Lundgren & Weiss, 1970; Haeusler & Finch, 1972; Finch, 1974a). Furthermore, these adaptive structural changes seem to be reversible in those animals with hypertension of a short duration (Weiss, Lundgren & Folkow, 1974; Lundgren, 1974b).

Some observations are more compatible with the view that individual smooth muscle cells of the hypertensive animals display a true supersensitivity and would therefore lead to a permanently increased vascular tone and presumably be the causal factor of the elevated blood pressure (Bandick & Sparks, 1970; Bohr & Sitrin, 1970; Holloway & Bohr, 1973).

The present studies were carried out on rats with a hypertension of long-standing duration and the reactivity was measured in the mesenteric bed. Even after prolonged lowering of the blood pressure by means of antihypertensive therapy, an increased reactivity was observed in vascular preparations from hypertensive animals. Preliminary results have been reported to the British Pharmacological Society (Finch, 1974b).

Methods

Male normotensive rats from a closed randomized colony (Bradford C.F.E.) were used for normotensive controls and for inducing deoxycorticosterone (DOCA)/NaCl hypertension.

Induction of experimental hypertension

Surgical procedures were carried out under ether anaesthesia in 6-7 week old animals. DOCA/NaCl hypertension was induced by subcutaneous implantation of DOCA acetate pellets (2 x 25 mg), unilateral nephrectomy and replacement of the drinking water with 0.9% w/v NaCl solution for a period of 4 weeks. Blood pressures of conscious animals were measured by an indirect tail cuff method. Rats with systolic blood pressures of at least 185 mmHg were considered hypertensive and those with a blood pressure between 110 and 140 mmHg were considered normotensive.

Long-term antihypertensive therapy

Eight weeks after the surgical procedures hypertensive rats and unoperated, age-matched normotensive rats were selected into groups for antihypertensive therapy. The animals were then given the drug combinations in the drinking water for a period of 10 weeks (Solution A: hydrallazine 0.1 g/litre + hydrochlorothiazide 0.25 g/litre + reserpine 5 mg/litre; Solution B: mecamylamine 0.05 g/litre + hydrallazine 0.05 g/litre). Blood pressures of treated and untreated groups were measured every 2 weeks and also measured within 48 h of setting up the perfused mesentery preparation.

Isolated perfused mesenteric artery preparations

Vascular reactivity to noradrenaline (base), 5-hydroxytryptamine (base) and adenosine 5′-triphosphate (salt) were determined in the isolated Krebs perfused mesenteric artery preparations by the method described previously (Haeusler & Finch, 1972). The perfusion experiments in treated animals were all carried out during the eleventh week after the antihypertensive therapy using double organ baths for paired experiments.

Statistical analysis

For evaluation of data, Student's *t* test was used. Throughout the paper, mean values are given together with the standard error of mean (s.e.); *n* is the number of estimations.

Drugs

The following drugs were used: adenosine 5′-triphosphate (ATP, Koch-Light), deoxycorticosterone acetate (Organon Laboratories); hydrallazine hydrochloride (Ciba Laboratories); hydrochlorothiazide (Merck, Sharpe & Dohme); 5-hydroxytryptamine creatinine phosphate (5-HT, Koch-Light); mecamylamine hydrochloride (Merck, Sharpe & Dohme); noradrenaline acid tartrate (Hoechst) and reserpine phosphate (Ciba Laboratories).

Table 1 Effect of long-term anti-hypertensive therapy on the resting systolic blood pressures of normotensive and chronic DOCA/NaCl hypertensive rats

	Before	*After*	*Treatment*
Normotensive	129 ± 6 (12)	122 ± 7 (12)*	Normal
Normotensive	124 ± 5 (10)	102 ± 7 (10)†	Solution A
Normotensive	127 ± 8 (10)	117 ± 8 (10)*	Solution B
Hypertensive	192 ± 9 (12)	188 ± 9 (9)*	Normal
Hypertensive	195 ± 11 (12)	130 ± 8 (8)†	Solution A
Hypertensive	203 ± 8 (12)	133 ± 9 (8)†	Solution B

Values indicate systolic blood pressures (mmHg ± s.e. mean) measured by indirect tail/cuff method. Antihypertensive therapy for 10 weeks. Drinking water of solution A contained hydrochlorothiazide 0.25 g/litre, reserpine 5 mg/litre and hydrallazine 0.1 g/litre. Solution B contained mecamylamine 0.05 g/litre and hydrallazine 0.05 g/litre.

* No significant differences. † $P < 0.05$.

Results

Effect of antihypertensive therapy on the resting blood pressures of normotensive and experimental hypertensive rats

The mean values of the resting blood pressures of normotensive and DOCA/NaCl hypertensive rats did not alter significantly during the 10 week period of the experiment when given normal drinking water (Table 1). In the normotensive rats treated with antihypertensive combination A, the blood pressures fell significantly (22 mmHg) whilst solution B produced only a moderate fall (10 mmHg) in normotensive animals. Both antihypertensive combinations (A and B) produced a marked fall in blood pressures in hypertensive animals and these were maintained within normotensive levels throughout the 10 week period of drug therapy (Table 1).

Vascular reactivity of the perfused mesenteric artery preparation from normotensive and hypertensive animals

Using the isolated perfused mesenteric artery preparation the vasoconstrictor responses to noradrenaline, 5-HT and ATP were determined in normotensive and hypertensive animals. Noradrenaline, 5-HT and ATP produced dose-dependent vasoconstrictor responses in preparations from both normotensive and hypertensive rats (Figures 1-3). In preparations obtained from hypertensive animals the dose-response curves produced by all three vasoconstrictor agents all exhibited a steeper slope and a higher maximum was obtained when compared with the curves from preparations of normotensive rats. However, the threshold doses of noradrenaline, 5-HT and ATP were not significantly different from those in normotensive controls (Figures 1-3). At the completion of each experiment higher doses of each vasoconstrictor agent were added to show that the responses were truly maximal.

In preparations obtained from normotensive rats treated with either antihypertensive solution A or B, the dose-response curves to noradrenaline, 5-HT and ATP were identical to those obtained in preparations from untreated normotensive rats (Figures 1-3). Using the preparations from 'hypertensive' rats in which the blood pressures had been normalized for a ten-week period by the use of antihypertensive therapy (solutions A and B), the dose-response curves for noradrenaline, 5-HT and ATP were almost identical to those obtained in preparations from age-matched hypertensive rats which had not received the antihypertensive therapies (Figures 1-3). The maximal responses to all three vasoconstrictor agents were also not significantly changed from those obtained with the untreated hypertensive rats.

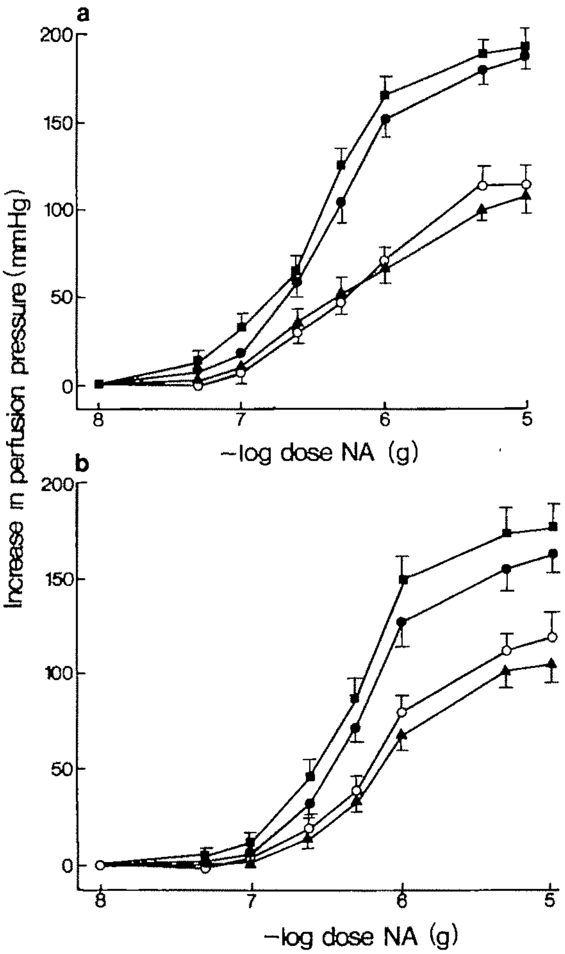

Figure 1 Dose-response curves for the vasoconstrictor effect of noradrenaline (NA) in isolated perfused mesenteric artery preparations: (a) from normotensive rats (○); normotensive rats after antihypertensive treatment A (▲), DOCA/NaCl hypertensive rats (●); DOCA/NaCl 'hypertensive' rats after antihypertensive treatment A (■); (b) from normotensive rats (○); normotensive rats after antihypertensive treatment B (▲); DOCA/NaCl hypertensive rats (●); DOCA/NaCl 'hypertensive' rats after antihypertensive treatment B (■) The mean values are shown; vertical bars indicate s.e. mean. Blood pressures before and after the 10-week treatment are shown in Table 1.

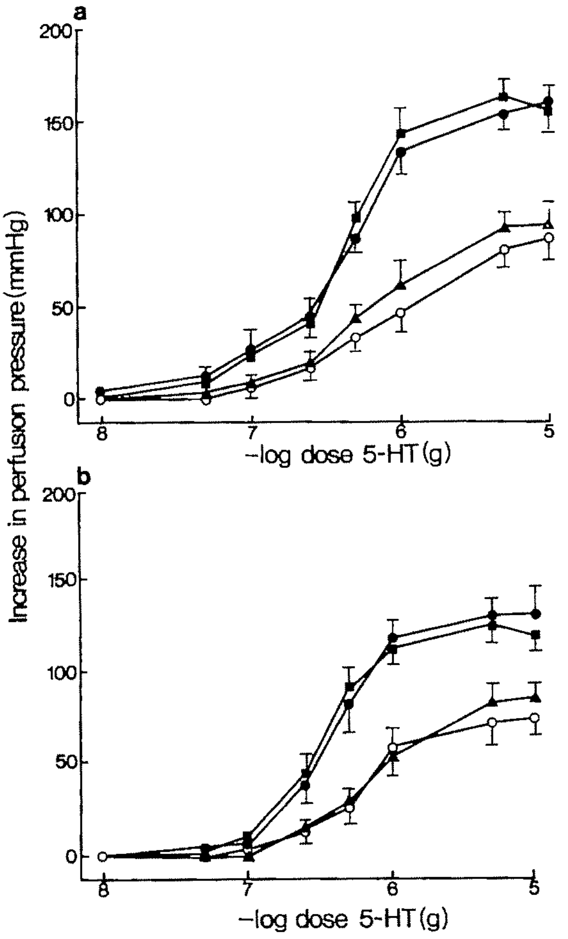

Figure 2 Dose-response curves for the vasoconstrictor effect of 5-hydroxytryptamine (5-HT) in isolated perfused mesenteric artery preparations: (a) from normotensive rats (○); normotensive rats after antihypertensive treatment A (▲); DOCA/NaCl hypertensive rats (●); DOCA/NaCl 'hypertensive' rats after antihypertensive treatment A (■). (b) from normotensive rats (○); normotensive rats after antihypertensive treatment B (▲); DOCA/NaCl hypertensive rats (●); DOCA/NaCl 'hypertensive' rats after antihypertensive treatment B (■). The mean values are shown; vertical bars indicate s.e. mean. Blood pressures before and after the 10-week treatment are shown in Table 1.

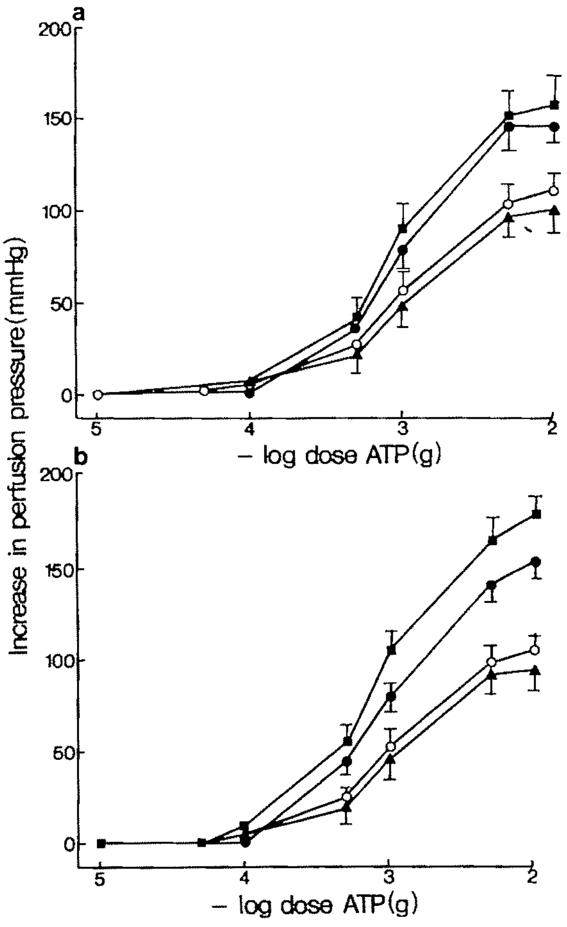

Figure 3 Dose-response curves for the vasoconstrictor effect of adenosine 5′-triphosphate (ATP) in the isolated perfused mesenteric artery preparations: (a) from normotensive rats (○), normotensive rats after antihypertensive treatment A (▲); DOCA/NaCl hypertensive rats (●); DOCA/NaCl 'hypertensive' rats after antihypertensive treatment A (■); (b) from normotensive rats (○); normotensive rats after antihypertensive treatment B (▲); DOCA/NaCl hypertensive rats (●); DOCA/NaCl 'hypertensive' rats after antihypertensive treatment B (■). The mean values are shown; vertical bars indicate s.e. mean. Blood pressures before and after the 10-week treatment are shown in Table 1.

Effect of antihypertensive therapy on the incidence of hypertensive lesions and heart weight in DOCA/NaCl hypertensive and normotensive rats

In order to discover whether the long-term antihypertensive treatment produced any reversal of the pathological states involved in DOCA/NaCl hypertension, heart weight (ventricles) and incidence of perarteritis nodosa of the mesentery was determined in all animals used for vascular reactivity studies. In normotensive rats the heart weights of a group of ten rats was found to lie

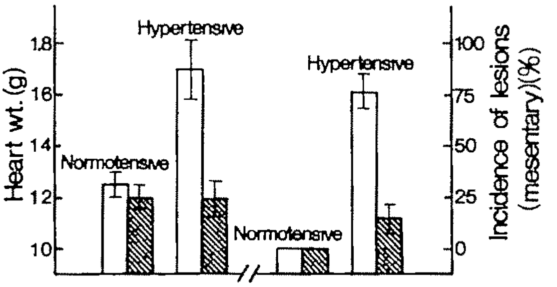

Figure 4 Effect of long term antihypertensive therapy on the incidence of arterial lesions and change in heart-weight in normotensive and DOCA/NaCl hypertensive rats Each column represents the mean value obtained from 10 animals. Hatched columns: effect of 10 weeks antihypertensive therapy (hydrochlorothiazide 0.25 g/litre, reserpine 5 mg/litre, hydrallazine 0.1 g/litre).

within a very small weight range (Figure 4). The age-matched DOCA/NaCl hypertensive rats (18 weeks post DOCA treatment/nephrectomy) exhibited a massive cardiac hypertrophy, the heart weight being increased by 50% compared with normotensive animals (Figure 4). This cardiac hypertrophy was completely reversed by 10 weeks of antihypertensive treatment with a combination of hydrochlorothiazide, reserpine and hydrallazine. In normotensive animals this antihypertensive therapy did not significantly change the heart weight compared with those from untreated normotensive animals.

In the mesentery of DOCA/NaCl hypertensive rats, with a hypertension of approximately 18 weeks duration, the incidence of lesions (perarteritis nodosa) was 75% whilst no lesions were seen in the age-matched normotensive animals (Figure 4). The long-term antihypertensive treatment almost completely reversed these lesions in the hypertensive rats with a normalized blood pressure (Table 1. Figure 4).

Discussion

An increased vascular reactivity of the perfused mesenteric artery preparation to various vasoconstrictor agents was demonstrated using 7-month old DOCA/NaCl hypertensive rats. These findings confirm earlier results with spontaneously hypertensive rats (Folkow *et al.*, 1970; Finch, 1974a), in DOCA/NaCl hypertensive rats (Beilin & Ziakas, 1972; Finch & Haeusler, 1974) and in renal hypertensive rats (Lundgren, 1974a, b). As in previous studies with spontaneously hypertensive rats the maximal responses obtained to ATP, or noradrenaline were higher than those to 5-HT (Finch, 1974a). The absence of any significant increased threshold response to any of the vasoconstrictor agents confirms previous work in many regions of the cardiovascular system (Haeusler & Finch, 1972; Finch, 1974a; Lundgren, 1974a, b; Weiss, 1974a, b), and is strong evidence for adaptive/structural changes, an encroachment of the lumen, and not any true hyperresponsiveness of the smooth muscle cells of hypertensive animals (Folkow *et al.*, 1970; Folkow, 1972). Even so, there are some experimental results in support of a true hyperresponsiveness of the excitation contraction coupling mechanism since in completely depolarized mesentery preparations there is no increased reactivity to calcium in preparations from hypertensive animals (Finch & Haeusler, 1974).

In DOCA/NaCl hypertensive animals in which the blood pressure had been elevated for approximately 8 weeks, it was found that with antihypertensive combinations of reserpine, hydrochlorothiazide and hydrallazine or hydrallazine and mecamylamine the blood pressures could be normalized for a 10-week period. These drug combinations have been shown to be effective in lowering the blood pressures in spontaneously hypertensive rats (Freis, Ragan, Pillsburg & Mathews, 1972).

The 10-week treatment with both antihypertensive combinations did not affect the vascular reactivity to ATP, 5-HT or noradrenaline in the mesentery preparations from normotensive animals which suggests that no impairment of receptors sites and/or uptake mechanism were involved. Furthermore, no pathological changes were observed in spontaneously hypertensive rats given a similar treatment for 6 months (Freis *et al.*, 1972). However, it was surprising to find that in the 'hypertensive' animals which had normalized blood pressures after the 10-week antihypertensive treatments, the vascular reactivity to 5-HT, ATP and noradrenaline was still increased and identical to that from untreated hypertensive animals. Similar results were obtained in a previous study in which antihypertensive treatment was carried out for only a 4-week period (Finch, 1974a).

In studies with both renal and spontaneously hypertensive rats adaptive/structural changes have been shown to take place rapidly in response to the elevated blood pressure (Folkow *et al.*, 1970; Lundgren, 1974a; Weiss & Hallbäck, 1974). Also these groups of workers have shown that a rapid regression takes place in younger hypertensive animals but found in the older established hypertensive rats that the blood pressures could not be normalized by some antihypertensive agents and therefore full regression of vascular design was not obtained (Weiss & Hallbäck, 1974; Weiss *et al.*, 1974).

Factors which may alter vascular reactivity during the prolonged hypertensive phase may include lesions of the vasculature. The incidence of these lesions in the mesentery and the marked cardiac hypertensive seen in these chronic DOCA/NaCl hypertensive animals was reversed by the antihypertensive treatment. Similar results have also been observed in spontaneously and renal hypertensive rats using antihypertensive agents such as α-methyl dopa and propranolol (Lundgren, 1974b; Sen, Tarazi, Khairallah & Bumpus, 1974; Weiss *et al.*, 1974). All these observations suggest that at least some of the secondary changes involved in experimental hypertension can be reversed by prolonged antihypertensive treatment.

The exact role of the increased reactivity to vasoconstrictor stimuli in experimental hypertension must remain in doubt. Although the preparations used in this study contained only arteries and arterioles, with no precapillary resistance vessels, and therefore cannot be compared directly with results using intact resistance vessels (Weiss, 1974a; Lundgren, 1974a, b), the preparations from 'hypertensive' animals still exhibited an increased reactivity even after a normalized blood pressure for a 10-week period. It is possible that the adaptive/structural changes, with an increased amount of supporting tissues containing elastin-collagen within the blood vessels, account for the higher maximal responses to vasoconstrictor agents and that no regression of this supporting tissue takes place. This hypothesis is strengthened by pathological studies in which the elastin-collagen deposition persists in the aortae of chronic hypertensive rats (Wolinsky, 1971, 1972). The regression of structural changes observed in 'true' resistance vessels may reflect a hypertrophy of the vessles, but this still depends on the extent of loading since it has been reported that chronic hypertensive animals become resistant to antihypertensive agents (Lundgren, 1974a).

In conclusion, the experimental results have shown that an increased reactivity to various vasoconstrictor stimuli occurs in blood vessels of the mesentery from chronic DOCA/NaCl hypertensive rats. Furthermore, this increased reactivity persists after the blood pressures had been normalized by antihypertensive treatment, suggesting that the hyperresponsiveness is only secondary to the hypertension.

The author is extremely grateful for the technical assistance of Miss J. West and Mr R. Goundry and for typing assistance by Miss A.L. Sanderson. The generous supply of drugs by Merck, Sharpe and Dohme (U.K.) Ltd. and Ciba (U.K.) Ltd. is also gratefully acknowledged.

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(Received February 18, 1975.
Revised April 19, 1975.)

Br. J. Pharmac. (1975), 54, 445-451

STUDIES ON THE HYPOTENSIVE ACTION OF α-METHYLDOPAMINE

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1 Intraventricular α-methyldopamine (50-200 μg) produced a dose-related fall in blood pressure in conscious spontaneously hypertensive rats. Pretreatment with intraventricular 6-hydroxydopamine prevented this hypotensive effect of α-methyldopamine.

2 The hypotensive effect of α-methyldopamine was prevented by intraventricular injection of phentolamine or desmethylimipramine, but not by intraperitoneal injection of haloperidol.

3 Pretreatment with U-14,624, a selective central dopamine-β-hydroxylase inhibitor, prevented the hypotensive effect of α-methyldopamine.

4 α-Methyldopamine was considerably less potent than noradrenaline as a pressor agent in the pithed rat, but noradrenaline and α-methylnoradrenaline were found to be equipotent.

5 α-Methyldopamine (1-5 mg i.c.v.) reduced pressor responses elicited by electrical stimulation of the midbrain reticular formation in cats anaesthetized with chloralose.

6 It is concluded that the hypotensive action of α-methyldopamine in conscious animals involves intact central α-adrenergic neurones and a central adrenergic uptake mechanism for the formation of α-methylnoradrenaline.

Introduction

Carlsson & Lindqvist (1962) suggested that decarboxylation products of α-methyldopa, α-methyldopamine (MDA) and α-methylnoradrenaline might take over the function of normal transmitters in the brain. Day & Rand (1963, 1964) extended this hypothesis to propose that α-methyldopa exerted its hypotensive effect by the formation of α-methylnoradrenaline which was assumed to be a less potent transmitter than noradrenaline in the peripheral sympathetic nervous system. However, there is evidence for only a moderate impairment of peripheral transmission (Haefely, Hurlimann & Thoenen, 1967; Henning & Svensson, 1968; Finch, 1971; Finch & Haeusler, 1973) and Trinker (1971) has reported that α-methylnoradrenaline and noradrenaline are equipotent in the anaesthetized dog and pithed rat.

Henning & Van Zwieten (1968) proposed a central site of action for α-methyldopa since the hypotensive effect of the drug was far greater when administered into the vertebral artery than when given intravenously, and other workers (Henning, 1969; Ingenito, Barrett & Procita, 1970; Heise & Kroneberg, 1973; Finch & Haeusler, 1973) have provided evidence supporting this view.

The present study provides evidence that the hypotensive effect of MDA involves stimulation of central α-adrenoreceptors and requires an intact central adrenergic uptake mechanism.

Methods

Measurement of arterial blood pressure in conscious spontaneously hypertensive rats

Spontaneously hypertensive rats (SHR) which originated from a hypertensive mutant of the Japanese strain (Roche Basle) by brother-sister mating, were used for these experiments. Rats were anaesthetized with chloral hydrate (200 mg/kg i.p.). A polypropylene cannula (Portex PP25 tip welded to a PP50 catheter) was introduced down the right carotid artery until the tip was positioned in the thoracic aorta, and the cannula was exteriorised at the back of the neck. An intraventricular cannula (Hayden, Johnson & Maickel, 1966) was inserted into the lateral cerebral ventricles via a trephine hole drilled 1 mm lateral and 1 mm posterior to the bregma and was fixed using dental acrylic cement. Blood pressures were recorded 1-2 days later in conscious unrestrained rats, with a Statham pressure transducer and a Devices M2 recorder. Drugs were injected intraventricularly (i.c.v.) in volumes not greater than 20 μl using a microlitre syringe with the needle cut down so that the tip extended 0.5 mm beyond the cannula guide into the lateral ventricles of the brain.

Pithed rat preparation

Pithed rat preparations were used to study the pressor response to noradrenaline, α-methylnoradrenaline and MDA injected intravenously.

Experiments on anaesthetized cats

Cats of either sex (2-3 kg) were anaesthetized with chloralose (60 mg/kg i.v.). The femoral artery and vein were then cannulated and a Statham pressure transducer connected to the arterial cannula. The animal's head was fixed in a David Kopf stereotaxic apparatus and a bipolar electrode introduced via a trephine hole in the skull into the midbrain reticular formation using the following co-ordinates: A = 5.0, L = 1.0-2.0, V = −0.5 (Snider & Niemer, 1961).

A trephine hole was drilled in the skull 7 mm posterior and 5 mm lateral to the bregma. Drugs were injected into the lateral cerebral ventricles with a microlitre syringe, the needle of the syringe penetrating 14 mm below the top of the skull.

At the end of each experiment, Trypan blue dye was injected through the ventricular cannulae and the brain examined for ventricular staining. Groups of 4-6 animals were used for these experiments.

Drugs

Desmethylimipramine hydrochloride (Ciba Geigy), haloperidol (Janssen), 6-hydroxydopamine hydrobromide (F. Hoffman La Roche, Basle), (±)-α-methyldopamine hydrobromide (synthesized by Janssen & Roche Products), (−)-methylnoradrenaline hydrochloride (Hoechst), (−)-noradrenaline acid tartrate (Hoechst), phentolamine mesylate (Rogitine, Ciba-Geigy) U-14,624 (1-phenyl-3-(2-thiazolyl)-2-thiourea) Aldrich chemicals). Noradrenaline, α-methylnoradrenaline, MDA and 6-hydroxydopamine refer to base weight, all other drugs refer to the salt. Haloperidol was dissolved in 1% lactic acid, U-14,624 was given by the intraperitoneal route injected as a suspension, 18 h before the experiments were performed. 6-Hydroxydopamine was administered to conscious rats by intraventricular route at 2 day intervals (3 x 250 μg) and experiments performed 7 days later. 6-Hydroxydopamine and MDA were dissolved immediately before use in 0.01 N HCl. α-Methylnoradrenaline and noradrenaline (for intravenous use) were stored in 0.1 N HCl and diluted in 0.9% w/v NaCl solution (saline) immediately before use. In the conscious rat experiments drugs for intraventricular administration were all dissolved in 0.01 N HCl immediately before use and the same volume of 0.01 N HCl was injected into the vehicle control animals.

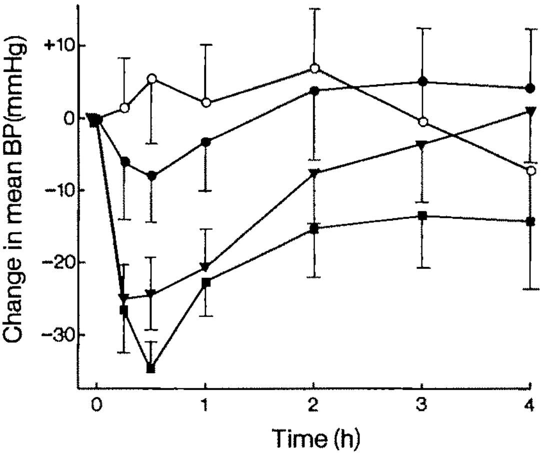

Figure 1 The effect of various doses of α-methyldopamine on the resting blood pressure of conscious, unrestrained spontaneously hypertensive rats. Vehicle of 20 μl 0.01 N HCl (○); intraventricular α-methyldopamine, 50 μg (●); 100 μg (▼); 200 μg (■). Vertical bars show the s.e. mean (*n* = 8 for all groups).

Results

Hypotensive action of α-methyldopamine in conscious hypertensive rats

Conscious spontaneously hypertensive rats were used in groups of 8 for this study. The mean arterial blood pressure was recorded directly via a catheter introduced into the aortic arch. MDA (50-200 μg i.c.v.) caused a dose-related fall in mean arterial blood pressure, the maximal fall being measured at 30 min (Figure 1). After the fall in blood pressure the pressure rose to values approaching control blood pressure values by the end of the 4 h experimental period.

An intraventricular injection of phentolamine (200 μg/rat) 1 h before the injection of MDA (150 μg i.c.v.) completely abolished the hypotensive action of intraventricular MDA (150 μg) (Table 1).

Administration of intraventricular 6-hydroxydopamine (3 x 250 μg) in doses shown to produce central sympathectomy and depletion of catecholamines (Uretsky & Iversen, 1970; Haeusler, Finch & Thoenen, 1972) greatly reduced

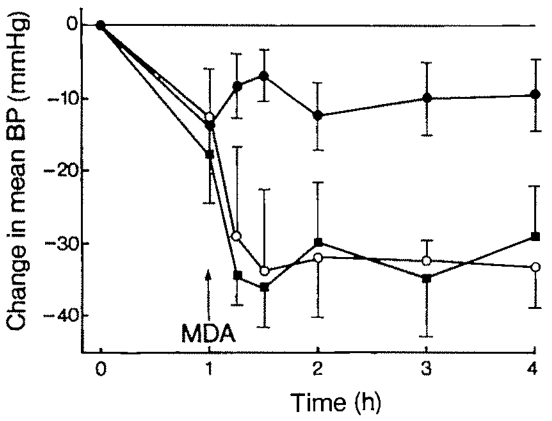

Figure 2 The effect of haloperidol on the hypotensive action of intraventricular α-methyldopamine (MDA) in conscious unrestrained spontaneously hypertensive rats. α-Methyldopamine (150 μg i.c.v.) in rats pretreated with vehicle (lactic acid 1% i.p.) (○); α-methyldopamine (150 μg i.c.v.) in rats pretreated with haloperidol (0.5 mg/kg i.p.) 1 h previously (■), haloperidol (0.5 mg/kg i.p.) alone (●). Vertical bars show s.e. mean. (n = 8 for all groups).

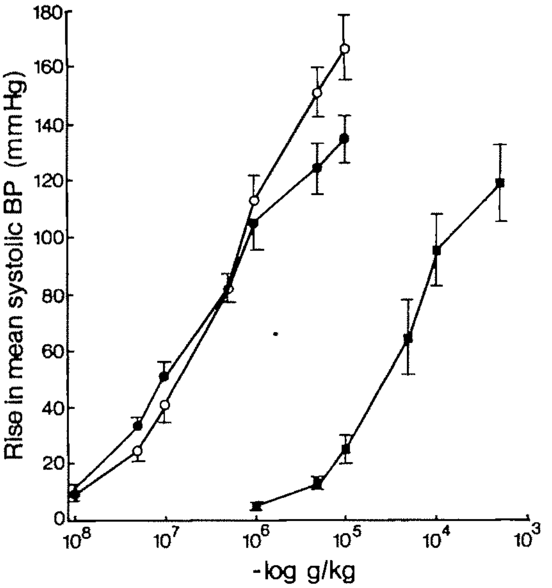

Figure 3 The pressor responses to intravenous noradrenaline (○), α-methylnoradrenaline (●) and α-methyldopamine (■) in pithed rats. The vertical bars show s.e. mean (n = 7 for all groups).

the hypotensive action of intraventricular MDA (150 μg) (Table 1).

Administration of intraventricular desmethylimipramine (200 μg/rat) 1 h before administration of MDA (150 μg i.c.v.) completely abolished the hypotensive action of MDA (Table 1).

In rats pretreated with U-14,624 (200 mg/kg i.p.) 18 h previously, a dose known to inhibit central dopamine-β-hydroxylase in the rat (Johnson, Boukma & Kim, 1970), the hypotensive action of the MDA (150 μg i.c.v.) was completely abolished (Table 1).

Administration of haloperidol (0.5 mg/kg i.p.) to rats 1 h before intraventricular injection of

Table 1 The hypotensive effect of intraventricular α-methyldopamine (MDA) in conscious spontaneously hypertensive rats modification by pretreatment with phentolamine, desmethylimipramine (DMI), 6-hydroxydopamine (6-OHDA) and U-14, 624

Treatment	(n)	Mean resting BP	Change in mean BP (mmHg) ± s.e. mean 0.25 h	0.5 h	1.0 h	2.0 h	3.0 h	4.0 h
0.01 NHCl vehicle i.c.v.	8	140.3	+1.3	+5.6	+2.2	+7.2	−0.3	−7.2
		±8.9	±7.1	±9.2	±8.0	±8.9	±6.4	±6.6
MDA (150 μg i.c.v.)	8	145.3	−24.1	−26.9	−13.8	−10.3	−8.5	−10.0
		±5 4	±7.3	±5.7	±6.5	±5.3	±8.4	±7.7
Phentolamine	8	141.3	+11.8	+20.7	+10.5	+22.1	+15.0	+17.1
(200 μg i.c.v.)		±13 2	±13.0	±10.1	±10.6	±12.1	±11 8	±10.3
MDA (150 μg i.c.v.) 1h	8	133.8	+8.4	+3.9	+1.2	+14.0	+9.0	+11.5
after phentolamine (200 μg)		±10.1	±9.3	±9.6	±11.2	±7.6	±5.8	±4.8
MDA (150 μg i.c.v.) 1 h	8	148.0	−1.0	+2.9	+1.9	0.3	−6.0	−4.2
after DMI (200 μg i.c.v.)		±7.6	±6.6	±7.4	±9.3		±6.8	±5.8
MDA (150 μg i.c.v.) after	8	142 0	+5.0	−3.8	−1.0	−5.0	+2.3	+5.2
6-OHDA (3 x 250 μg i.c.v.)		±6.9	±5 0	±7.6	±8.3	±7.4	±9.4	±10.6
MDA (150 μg i.c.v.) 18 h	8	138.2	−4.3	−7.5	−3.1	+2.7	−2.8	+10.1
after U-14, 624 (200 mg/kg i.p.)		±6.8	±6.7	±6.1	±5.8	±6.4	±7.0	±7.8

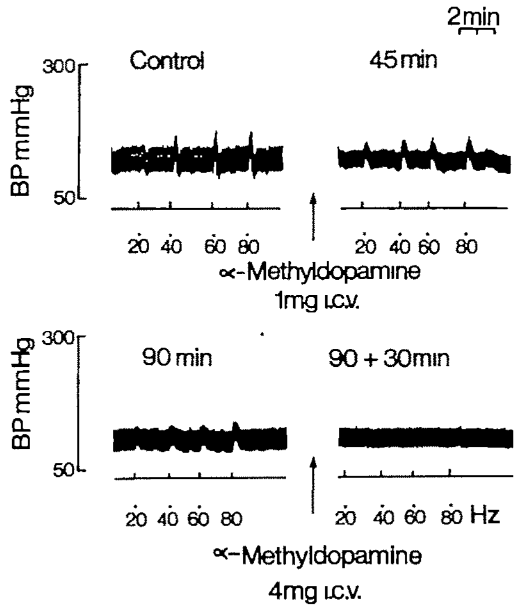

Figure 4 The effects of intraventricular α-methyldopamine (MDA) on the hypertensive action of stimulation of the mid brain reticular formation of a cat anaesthetized with chloralose. Stimulations were carried out for periods of 10 s at an intensity of 30 V, 0.1 ms duration at 20, 40, 60 and 80 Hz. Following control responses, 1 mg MDA was injected; after 90 min a further dose of 4 mg was injected.

MDA (150 μg i.c.v.) failed to alter the hypotensive action of MDA (Figure 2). However, the lactic acid (1%) solvent of haloperidol, produced a prolonged fall in blood pressure (10 mmHg) in all animals.

Pressor effects of noradrenaline, α-methylnoradrenaline and α-methyldopamine in pithed rats

The pressor effect of these three catecholamines was studied in pithed rats. Noradrenaline and α-methylnoradrenaline were found to be equipotent, whilst MDA was approximately 100 times less potent than noradrenaline (Figure 3) and the maximum pressor response was less than that for noradrenaline.

Effect of α-methyldopamine on centrally induced pressor responses in chloralose-anaesthetized cats

Electrical stimulation of the midbrain reticular formation in cats anaesthetized with chloralose induced an immediate rise in blood pressure. Graded responses were obtained by increasing the frequency of stimulation and maximal responses were observed at 80 Hz. Intraventricular MDA (1 mg + 4 mg cumulative), caused the responses to be reduced and then abolished after a period of 120 min (Figure 4). No reduction in these responses to electrical stimulation occurred when the vehicle alone (20 μl 0.01 N HCl) was injected, and stimulations carried out for a similar time period.

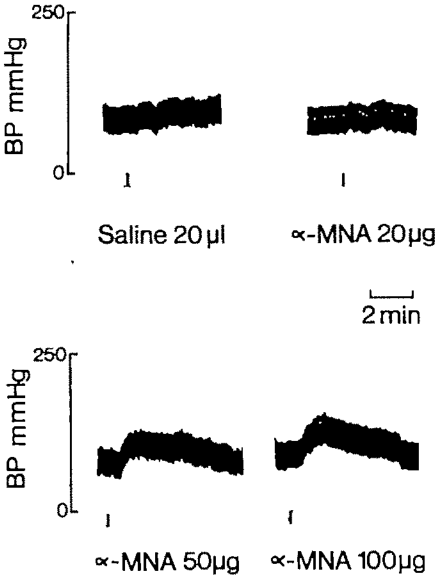

Figure 5 The effect of various doses of α-methylnoradrenaline (α-MNA) administered intraventricularly on the resting blood pressure of a cat anaesthetized with chloralose. No change in blood pressure was observed with the vehicle (20 μl saline).

Effect on intraventricular α-methylnoradrenaline in chloralose-anaesthetized cats

Intraventricular injections of α-methylnoradrenaline (20-100 μg) gave dose-related rises in blood pressure with no ensuing depressor response, even during a 3 h recording period (Figure 5).

Discussion

The results of the present study demonstrate the ability of intraventricular MDA to lower the blood pressure in conscious spontaneously hypertensive rats. This hypotensive action of intraventricular MDA was prevented by intraventricular pretreatment with 6-hydroxydopamine in doses known to cause extensive depletion of central catecholamines (Uretsky & Iversen, 1970; Haeusler *et al.*, 1972). Pretreatment with intraventricular desmethylimipramine, which prevents uptake of catecholamines into central adrenergic neurones (Carlsson, Fuxe, Hamberger & Lindqvist, 1966) or intraventricular phentolamine also prevented the hypotensive action of MDA. These results strongly suggest that intact central α-adrenergic neurones are involved in the hypotensive action of MDA, and that uptake of MDA into these neurones is a prerequisite for this action. β-Hydroxylation of MDA to α-methylnoradrenaline is also essential, since inhibition of central dopamine-β-hydroxylase by U-14,624 (Johnson *et al.*, 1970) also prevents the hypotensive action of MDA.

It is of interest that at higher doses (200 μg i.c.v.) MDA induced characteristic dopaminergic behavioural effects (sniffing and gnawing) suggesting a measure of dopamine receptor stimulation. Although dopaminergic pathways are believed to be involved in the central regulation of blood pressure (Bolme, Fuxe & Lidbrink, 1972) haloperidol, which is known to inhibit dopamine-induced stereotype and gnawing behaviour (Janssen, Niemegeers & Schellekens, 1965) did not antagonize the hypertensive action of MDA.

These results are in agreement with the hypothesis of Henning & Rubenson (1971) that the hypotensive action of α-methyldopa is mediated via α-methylnoradrenaline acting at central α-adrenoceptors. The hypotensive action of α-methyldopa has also been found to be antagonized by central α-adrenoceptor blockade, adrenergic uptake or 6-hydroxydopamine treatment in animals (Finch & Haeusler, 1973; Kale & Satoskar, 1970) and by tricyclic antidepressant treatment in man (White, 1965).

Injection of α-adrenoceptor agonists into ventricles of the brain may cause either pressor or depressor responses, possibly reflecting the site of interaction of the drugs with the neurones in the brain (Gagnon & Melville, 1966; Heise & Kroneberg, 1973; Correa & Graeff, 1974; Sinha & Schmitt, 1974; Day & Roach, 1974). In our experiments α-methylnoradrenaline given intraventricularly in cats anaesthetized with chloralose caused pressor responses with no associated fall in blood pressure; similar results were observed when catecholamines were injected directly into the posterior hypothalamus (Philippu, Przuntek, Heyd & Burger, 1971). Electrical stimulation of different areas of the brain similarly causes pressor or depressor responses. Stimulation of the midbrain reticular formation, posterior hypothalamus or locus coeruleus (Haeusler & Finch, 1972; Finch & Haeusler, 1973; Przuntek & Philippu, 1973) causes a rise in blood pressure. MDA reduced the pressor responses elicited by stimulation of the midbrain reticular formation, and also of the posterior hypothalamus (Finch, Hersom & Hicks, unpublished observations). It is a reasonable hypothesis that α-methyldopa and MDA may reduce these elicited pressor responses via α-methylnoradrenaline acting at central α-adrenoceptors, since Finch & Haeusler (1973) have shown the pretreatment with FLA-63, a dopamine-β-hydroxylase inhibitor abolished this inhibitory effect of α-methyldopa. Also clonidine partially inhibited the pressor response elicited by stimulation of the posterior hypothalamus (Haeusler & Finch, 1972; Haeusler, 1973). The mode of action of MDA and α-methyldopa in reducing centrally evoked pressor responses is still to be elucidated since Haeusler (1974) has reported that an increased sympathetic nerve activity after stimulation of the posterior hypothalamus could still be elicited, even when brain noradrenaline content had been depleted by reserpine and α-methylpara-tyrosine to 1-3% of normal. Therefore, non-noradrenergic neurones may be involved in this efferent pathway down to the preganglionic fibres.

Although the body of evidence is overwhelmingly in favour of a central locus of action for α-methyldopa and MDA, there is evidence supporting a peripheral false transmitter hypothesis. In the pithed rat, noradrenaline and α-methylnoradrenaline were found to be equipotent as pressor agents. However, MDA was considerably less potent than noradrenaline as a pressor agent and it is possible that this might reflect partially, a false transmitter action of MDA in the periphery. Finch (1971) could not show any impairment of the entire sympathetic outflow in the pithed rat by acute administration of α-methyldopa, but Finch & Haeusler (1973) found that α-methyldopa pretreatment reduced the vasoconstrictor responses to stimulation of the renal artery preparation, and that α-methylnoradrenaline had one eighth the potency of noradrenaline on this preparation. Recently Doxey & Scutt (1974) have shown that 4 pretreatments with α-methyldopa reduced the pressor responses to stimulation of the entire sympathetic outflow in the pithed rat, and reduced the positive chronotropic effects by stimulation of the cardiac nerves. Salmon & Ireson (1970) also claimed evidence for a short lasting peripheral impairment in the anaesthetized rat after α-methyldopa. Farmer (1965) also demonstrated impairment of the cat nictitating membrane after α-methyldopa.

In conclusion, MDA has been shown to exert a central control of the resting blood pressure in conscious animals. The results suggest that the hypotensive action of MDA involves intact central α-adrenergic neurones and a central adrenergic uptake mechanism for the formation of α-methylnoradrenaline.

The authors are extremely grateful to Roche Products and Janssen Pharmaceutical for generous donations of α-methyldopamine. This work was supported by a grant from the S.R.C. for studentships (A. Hersom and P. Hicks). The technical and typing assistance of Mr R. Goundry, Mrs C. Taylor and Miss A. Sanderson is gratefully acknowledged.

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(Received March 5, 1975)

Br. J. Pharmac. (1975), 54, 453-462

CARDIOVASCULAR DEPRESSANT EFFECTS OF NEOMYCIN AND GENTAMICIN IN RHESUS MONKEYS

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1 The acute cardiovascular effects of neomycin and gentamicin, representative aminoglycoside antibiotics, were examined in surgically-prepared anaesthetized rhesus monkeys.

2 Intravenous administration of 14, 28, and 56 mg/kg of neomycin consistently induced a dose-dependent depression of systemic blood pressure, cardiac output, left ventricular contractile force, maximum d*F*/d*t* of left ventricular contraction, and heart rate. Neomycin produced similar cardiovascular depressant effects when heart rate was maintained constant by electrical pacing.

3 Maximum depression of haemodynamic values usually occurred within 2 to 5 min after injection of neomycin; values then gradually returned to control levels within 20 to 30 (14 mg/kg) or 60 to 80 (56 mg/kg) minutes.

4 Injection of $CaCl_2$ (1.35 mEq Ca^{2+}/kg, i.v.) during the peak depressant effect of neomycin produced a rapid and maintained restoration of cardiovascular function to control levels; conversely, noradrenaline (2 μg, i.v.) or isoprenaline (0.5 μg, i.v.) produced only transient reversal of the neomycin effects.

5 Similar evidence of cardiovascular dysfunction was observed with gentamicin.

6 These findings demonstrate the direct cardiovascular depressant effects of aminoglycoside antibiotics in a higher primate species, and suggest that this adverse response is related to an alteration of calcium ion function.

Introduction

Although cardiovascular depression is infrequently recognized as an untoward side effect of the aminoglycoside antibiotics, several instances of adverse circulatory changes have been observed during therapy with representatives of this group of agents. Hypotensive episodes, for example, have occurred during treatment with gentamicin (Warner & Sanders, 1971; Hall, McGibbon, Evans & Meadows, 1972), streptomycin (Fisk, 1961, see Pittinger, Eryasa & Adamson, 1970), kanamycin (Ream, 1963), and neomycin (Pittinger, Long & Miller, 1958). Cardiovascular collapse has also been observed subsequent to exposure to aminoglycoside antibiotics (Ream, 1963; see Pittinger *et al.*, 1970). However, these adverse haemodynamic responses were not attributed to direct cardiovascular depressant effects of the antibiotics involved. Instead, cardiovascular changes have been unexplained or ascribed as secondary to bacteraemia (Hall *et al.*, 1972) or to the well-known respiratory depressant effects of these agents (Ream, 1963; Pittinger *et al.*, 1970).

It seems, therefore, that cardiovascular dysfunction may be an adverse side effect of the aminoglycoside antibiotics, but the potential for this activity is usually overlooked. Furthermore, few experimental studies have examined the direct haemodynamic effects of this extensively used group of drugs, and apparently no attempts have been made to investigate the mechanism of the cardiovascular effects of an aminoglycoside antibiotic. Present studies were undertaken in rhesus monkeys in order to examine *in vivo* the acute cardiovascular effects of typical aminoglycoside antibiotics, neomycin and gentamicin, in a higher primate species, and to determine the influence of Ca^{2+} and other inotropic agents on these responses.

Methods

The primates used in this study were young male rhesus monkeys (*Macaca mulatta*) weighing 4.0 to 6.0 kg. Monkeys were procured from a primate importer after a minimum 30-day post-capture holding period. After arrival at our facilities, the monkeys were placed on a minimum 60-day health-conditioning programme to assure that only monkeys in good health were used in an experiment. During this period, tuberculosis testing and deworming were carried out; however, care was taken to assure that monkeys were not exposed to drugs or environmental chemicals for at least 20 days before their use in an experiment.

Food was withheld for 14 to 16 h, and a monkey was then anaesthetized with pentobarbitone, 28 to 35 mg/kg administered intraperitoneally. During an experiment, supplementary doses of pentobarbitone were administered periodically in order to maintain a relatively constant depth of anaesthesia. The trachea was cannulated and positive pressure ventilation was maintained with a Harvard small-animal respirator. The right femoral artery was catheterized for monitoring of systemic blood pressure with a Statham P23DC transducer, and the ipsilateral femoral vein was catheterized to facilitate injection of drugs. The thoracic cavity was entered at the left 4th intercostal space, the lung was retracted dorsally with a moistened gauze sponge, and the pericardium was incised longitudinally.

The ascending aorta was carefully isolated and a Narco Bio-Systems, Inc., precalibrated electromagnetic flow probe (6-8 mm internal diameter) was placed around the aortic root. This flow probe was connected to a Narco Bio-Systems, Inc., electromagnetic flow meter that converted velocity of aortic blood flow to volume flow which was expressed as cardiac output (minus coronary flow) in ml/minute. Left ventricular contractile force was monitored by use of a Walton-Brodie strain gauge arch sutured to the left ventricular free wall, and contractile force was assessed in mm deflection of the recording instrument. The contractile signal was differentiated electronically (Grass 7P20) to obtain the first derivative of left ventricular contraction (maximum dF/dt), and this value was also assessed in mm deflection of the recording device. Heart rate was measured with a Grass 7P4D cardiotachometer that was triggered by either the electrocardiogram or the ventricular strain gauge deflection. Where indicated, heart rate was maintained constant by electrically pacing the heart with a Grass S-44 stimulator via needle electrodes placed in the left auricle. All parameters were recorded on a Grass 7B polygraph multichannel recorder. After the surgical procedures were completed, a 20 to 30 min stabilization period was allowed before the injection of any drug.

The sulphate salts of gentamicin (Gentamicin Reagent Solution, Schering Corp.) and neomycin (Sigma Chemical Co.) were used; dosages refer to the active antibiotic base. The gentamicin was a commercial reagent solution with no designated additives. Neomycin was dissolved in 0.9% w/v NaCl solution (saline); injections of equal volumes of saline produced no discernible effects on measured cardiovascular functions.

Initially, neomycin was injected intravenously in order to determine a dose-response relationship to the maximum cardiovascular effects produced by 14, 28, and 56 mg/kg of the antibiotic. Injections were usually made at 45 to 80 min intervals, but time was allowed in each case for measured parameters to return to control ± 10% of control levels and to stabilize before another injection was made. In three other monkeys, the effects of neomycin were determined both when heart rate was maintained constant by electrical stimulation and when heart rate was not paced. If the same dose of neomycin was administered to an individual monkey more than once, the response of that monkey to the dose was calculated as the average value.

In experiments that were designed to examine reversal of the neomycin effects, responses to the maximum dose of the antibiotic (56 mg/kg) were measured over a 1 h post-injection period. Reversal of cardiovascular depressant effects of neomycin by Ca^{2+} ($CaCl_2$, 10 mg/kg; 1.35 mEq Ca^{2+}/kg), noradrenaline (2 μg, total dosage), or isoprenaline (0.5 μg, total dosage) was determined by injection of one of these agents intravenously 2 to 3 min (2.5 ± 0.5) after the administration of 56 mg/kg of neomycin. The temporal relationship of recovery from cardiovascular depression seen after intervention with Ca^{++}, noradrenaline, or isoprenaline was compared to recovery seen after the administration of only 56 mg/kg of neomycin. Experiments were also conducted to examine the influence of Ca^{2+} on gentamicin-induced cardiovascular depression.

In several monkeys, control haemodynamic responses to Ca^{2+} ($CaCl_2.2H_2O$, 10% solution in distilled water), noradrenaline ((−)-noradrenaline hydrochloride), and isoprenaline ((−)-isoprenaline hydrochloride) were obtained before the administration of an antibiotic.

All values are expressed as the mean ± one standard error of the mean and the difference between two means was examined statistically by use of Student's t-test.

Results

Intravenous administration of neomycin consistently produced a dose-related depression of systemic blood pressure, heart rate, cardiac output, left ventricular contractile force, and dF/dt in open-chest rhesus monkeys. Typical

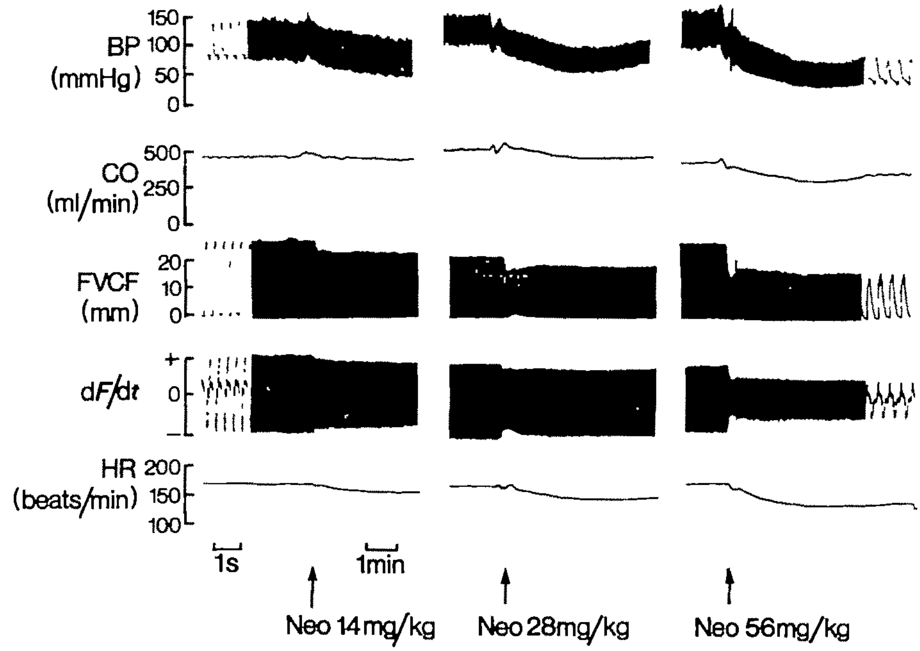

Figure 1 Cardiovascular depressant effects of 14, 28, and 56 mg/kg of neomycin intravenously in open-chest rhesus monkeys. BP = blood pressure; CO = cardiac output; LVCF = left ventricular contractile force; d*F*/d*t* = differentiated LVCF; HR = heart rate; Neo = neomycin.

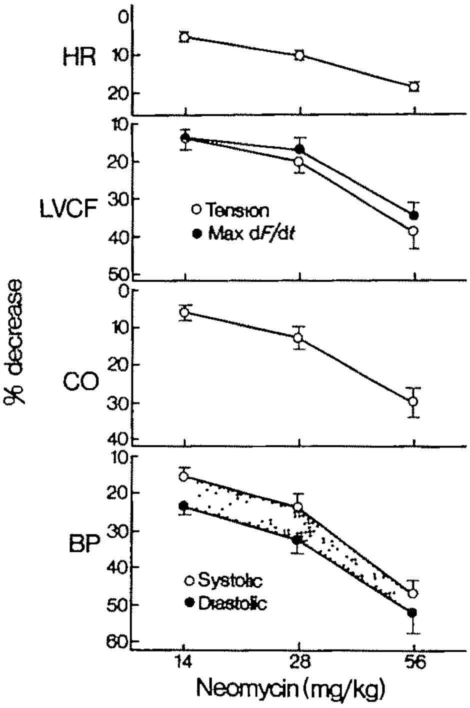

Figure 2 Dose-related cardiovascular depressant effects of intravenously administered neomycin in open-chest rhesus monkeys. Each value is the mean ± s.e. of 7 to 11 responses obtained from 7 to 9 monkeys, except cardiac output where $n = 6$. If an individual monkey was given the same dose of neomycin more than once, the average response was determined and used in calculating the mean response of the population to that dosage. BP = blood pressure; CO = cardiac output; HR = heart rate; LVCF = left ventricular contractile force.

responses of the cardiovascular system to the three doses of neomycin studied, 14, 28, and 56 mg/kg, are shown in Figure 1; absolute values of measured cardiovascular parameters are presented in Table 1; and Figure 2 displays the average percent depression of cardiovascular function produced by neomycin. With each dose of neomycin, maximum depression of the measured parameters usually occurred within 2 to 5 min after injection. Cardiovascular function then gradually returned to control or near control levels within 20 to 30 min after the lowest dose of neomycin (14 mg/kg) and usually within 60 to 80 min following injection of the largest dose (56 mg/kg) of the antibiotic.

Although the negative inotropic effects produced by each dose of neomycin appeared to be more pronounced than corresponding negative chronotropic responses (Figure 2), the possibility existed that tension changes in the myocardium were secondarily influenced by the neomycin-induced relative bradycardia. This aspect was examined in three additional monkeys by determining the effects of neomycin both when heart rate was maintained constant and when the heart was not paced. Under these conditions, blood pressure and generated tension in the myocardium seemed to be variably less affected by neomycin when the heart was paced; and maximum d*F*/d*t* of left ventricular contraction was slightly less depressed by all three doses of neomycin when heart rate was maintained constant (Figure 3). Although the small number of paced-nonpaced experiments preclude valid statistical evaluation, these examples illustrate that there seem to be minimal differences in the cardiovascular effects of neomycin during paced and nonpaced heart rate conditions.

The extent of cardiovascular depression produced by 56 mg/kg of neomycin was measured at various time intervals for 60 min after injection.

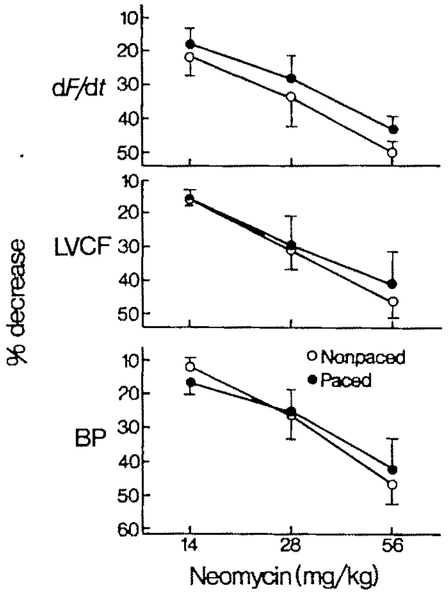

Figure 3 Effects of neomycin on blood pressure, left ventricular contractile force, and maximum d*F*/d*t* of left ventricular contraction in rhesus monkeys during paced and nonpaced heart rate conditions Neomycin was injected in the same monkeys on a random schedule during electrical pacing of heart rate (●) and when heart rate was not paced (○). Effects of neomycin 14 and 56 mg/kg were examined in 3 monkeys; 28 mg/kg was examined in 2 of the monkeys. BP = systolic blood pressure; d*F*/d*t* = maximum d*F*/d*t* of LVCF; LVCF = left ventricular contractile force.

Table 1 Cardiovascular effects of neomycin in rhesus monkeys

Neomycin	*Function*	*Pre-neomycin*	*Post-neomycin*	*Difference*	P*
14 mg/kg	Systolic BP (mmHg)	127.7 ± 8.4	108.1 ± 8.6	19.7 ± 2.9	<0.005
	Diastolic BP (mmHg)	74.5 ± 6.7	57.3 ± 5.9	17.2 ± 2.5	<0.005
	LVCF	23.5 ± 1.3	20.3 ± 1.4	3.1 ± 0.4	<0.005
	d*F*/d*t*	11.0 ± 0.9	9.9 ± 0.9	1.5 ± 0.2	<0.05
	CO (ml/min)	493.3 ± 26.9	463.3 ± 29.0	30.4 ± 6.9	<0.05
	HR (beats/min)	163.0 ± 9.7	153.6 ± 9.4	9.4 ± 1.7	<0.01
28 mg/kg	Systolic BP (mmHg)	118.8 ± 8.4	90.2 ± 9.0	28.6 ± 4.1	<0.001
	Diastolic BP (mmHg)	67.4 ± 4.8	45.2 ± 4.3	22.2 ± 3.1	<0.001
	LVCF	24.1 ± 1.8	19.7 ± 1.4	4.5 ± 1.0	<0.01
	d*F*/d*t*	10.2 ± 0.9	8.6 ± 0.9	1.9 ± 0.3	<0.05
	CO (ml/min)	536.0 ± 22.3	471.0 ± 15.4	65.0 ± 15.8	<0.01
	HR (beats/min)	163.5 ± 11.1	146.7 ± 11.6	16.8 ± 2.0	<0.005
56 mg/kg	Systolic BP (mmHg)	127.7 ± 9.9	69.3 ± 10.4	58.4 ± 4.8	<0.001
	Diastolic BP (mmHg)	74.7 ± 7.2	36.2 ± 6.7	38.5 ± 5.3	<0.001
	LVCF	25.2 ± 1.0	15.3 ± 1.2	9.9 ± 1.2	<0.001
	d*F*/d*t*	10.8 ± 1.1	6.9 ± 0.8	3.6 ± 0.6	<0.005
	CO (ml/min)	545.6 ± 37.3	398.8 ± 46.6	146.9 ± 17.1	<0.001
	HR (beats/min)	160.5 ± 8.1	130.6 ± 6.9	29.9 ± 2.4	<0.001

Each value is the mean ± s.e of 6-9 responses.

* Paired *t*-test for the neomycin response and corresponding control value. LVCF = left ventricular contractile force; d*F*/d*t* = differentiated LVCF; CO = cardiac output; HR = heart rate; for additional information, see legends for Figure 2 and 4.

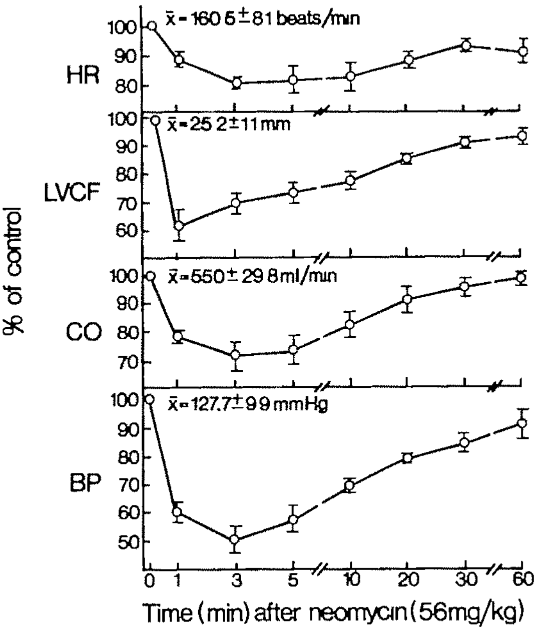

Figure 4 Recovery from the cardiovascular depressant effects of 56 mg/kg of neomycin in open-chest rhesus monkeys. Each value is the mean ± s.e. of 7 to 11 responses obtained from 7 to 9 monkeys, except cardiac output where $n = 6$. If an individual monkey was given the same dose of neomycin more than once, the average response was determined and used in calculating the mean response of the population to that dosage. BP = systolic blood pressure; CO = cardiac output; HR = heart rate; LVCF = left ventricular contractile force.

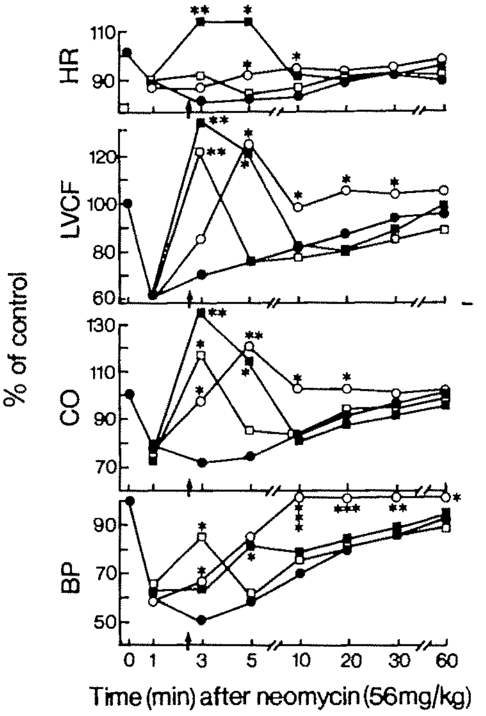

Figure 5 Antagonism of the cardiovascular depressant effects of neomycin (56 mg/kg) by calcium chloride, noradrenaline, or isoprenaline in rhesus monkeys. BP = systolic blood pressure; CO = cardiac output; HR = heart rate; LVCF = left ventricular contractile force (●) neomycin 56 mg/kg; (○) neomycin + $CaCl_2$ 10 mg/kg (1.35 mEq/kg Ca^{2+}); (■) neomycin + isoprenaline 0.5 µg; (□) neomycin + noradrenaline 2µg. Ca^{2+} ($n = 4$-5), noradrenaline ($n = 3$-4), or isoprenaline ($n = 4$-5) was injected, intravenously at arrow. Control neomycin curve ($n = 7$-9) was obtained from Figure 4.

* $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$; values are significantly greater than corresponding neomycin control values.

Values are shown in Figure 4, and they indicate the gradual recovery of haemodynamic parameters during the period. These curves of mean values will be displayed as control curves in several subsequent tracings to facilitate comparison of the antagonism of the effects of neomycin by noradrenaline, isoprenaline, and Ca^{2+}.

The administration of $CaCl_2$ 10 mg/kg (1.35 mEq Ca^{2+}/kg) during the peak depressant effects produced by 56 mg/kg of neomycin rapidly restored physiological values to control or near control levels (Figure 5). Within 2 to 7 min after administration of Ca^{2+}, systemic blood pressure, left ventricular contractile force, and cardiac output were similar to values seen prior to the administration of the antibiotic. Antagonism by Ca^{2+} was a maintained effect since cardiovascular function remained near control levels following the administration of this cation. Antagonism by Ca^{2+} of the depressant effects of neomycin on myocardial contraction, cardiac output, and blood pressure was statistically significant ($P < 0.05$ to $P < 0.0005$) at virtually all time intervals examined (Figure 5); antagonism of the negative chronotropic effects of neomycin by Ca^{2+} was less reliable (Figure 5).

In contrast to the effects seen with Ca^{2+}, noradrenaline and isoprenaline did not produce sustained antagonism of the neomycin effects (Figure 5). Both of these agents rapidly restored left ventricular contractile force and cardiac output to control or greater-than-control levels; however, this antagonism was of short duration and these parameters returned to depressed levels within a few minutes (Figure 5). Noradrenaline

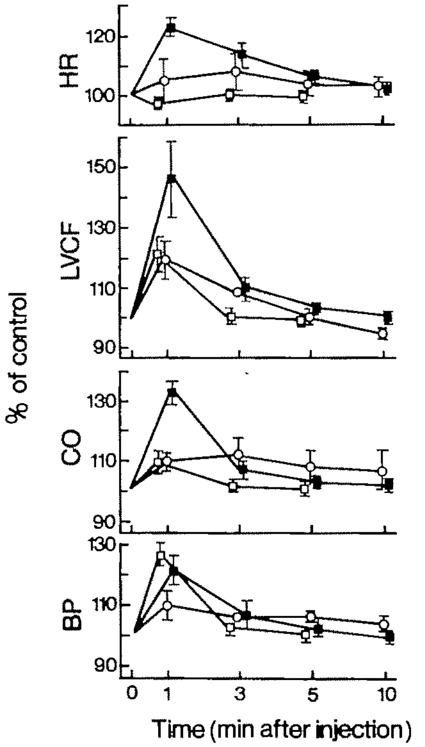

Figure 6 Cardiovascular effects of intravenously administered $CaCl_2$ (1.35 mEq Ca^{2+}/kg) (○), noradrenaline (2 µg) (□), and isoprenaline (0.5 µg) (■) in open-chest rhesus monkeys. Each value is the mean ± s.e. of 3 to 5 responses obtained from 3 or 4 monkeys. For abbreviations, see Figures 1-3.

and isoprenaline each produced a transient partial reversal of neomycin-induced hypotension. Isoprenaline produced a pronounced increase in heart rate whereas noradrenaline had minimal effect on heart rate in the presence of neomycin. The effects of noradrenaline and isoprenaline on measured parameters were not reliably different ($P > 0.05$ to $P > 0.6$, Figure 5) from control values seen with neomycin at 10, 20, 30 and 60 min after injection of the antibiotic.

Noradrenaline, isoprenaline and Ca^{2+} produced pronounced increases in blood pressure, cardiac output, and left ventricular contractile force in control monkeys that had not received an antibiotic (Figure 6). Isoprenaline and Ca^{2+}, but not noradrenaline, also increased heart rate (Figure 6). Physiological values returned to control levels within 10 min after injection of these inotropic agents (Figure 6).

In additional experiments, gentamicin produced cardiovascular depressant effects similar to that seen with neomycin. For example, intravenous administration of 30 to 40 mg/kg of gentamicin (37.5 ± 2.5 mg/kg; $n = 4$) produced a reduction of: systolic blood pressure from 136.0 ± 3.6 to 93.0 ± 7.5 mmHg (31.8 ± 4.0% decrease); diastolic blood pressure from 76.0 ± 1.0 to 47.0 ± 2.5 mmHg (38.1 ± 3.3% decrease); left ventricular contractile force from 25.2 ± 1.9 to 12.0 ± 1.8 mm (53.1 ± 3.9% decrease); d*F*/d*t* from 11.0 ± 0.6 to 5.8 ± 0.8 mm (48.3 ± 4.4% decrease); cardiac output from 452.5 ± 11.8 to 335.0 ± 20.2 ml/min (25.4 ± 6.2% decrease); and heart rate from 196.2 ± 2.4 to 173.0 ± 6.4 beats

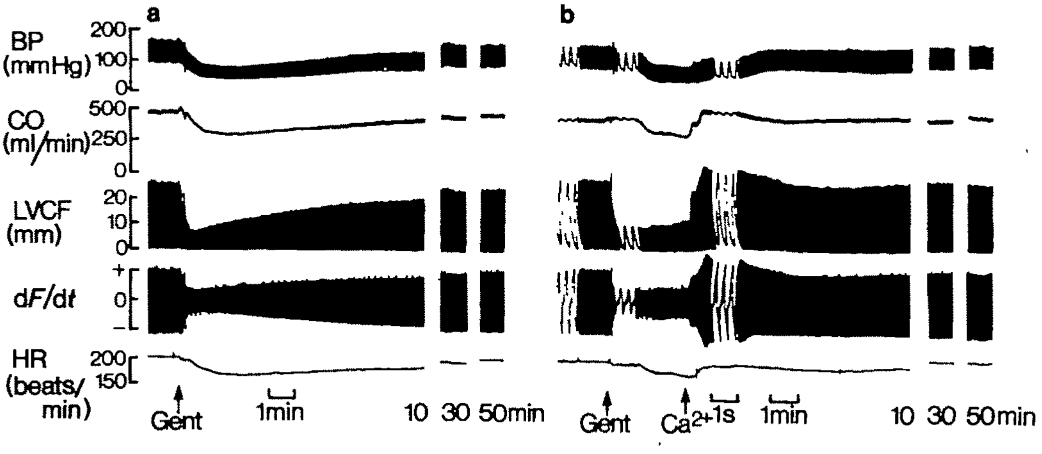

Figure 7 Cardiovascular depressant effects of gentamicin and reversal of gentamicin effects by calcium chloride in a rhesus monkey. (a) Typical responses to i.v. gentamicin (Gent, 40 mg/kg); (b) reversal of gentamicin (40 mg/kg) effects by intravenous $CaCl_2$ (1.35 mEq Ca^{2+}/kg). Agents were injected at arrows; 10, 30, and 50 min refer to time after injection of gentamicin. For abbreviations, see Figures 1-3.

per minute (11.8 ± 3.1% decrease). Ca^{2+} administration rapidly antagonized the depressant effects of gentamicin in rhesus monkeys and produced a maintained restoration of cardiovascular function to control values (Figure 7).

Discussion

Intravenous antibiotic therapy is often a routine procedure for prophylaxis or treatment of bacterial infections that may occur secondary to cardiovascular dysfunction (Rosenblum & Frieden, 1972). However, potential haemodynamic changes that may be elicited by antibiotics themselves are rarely considered when adverse cardiovascular experiences are examined for aetiological factors. For example, hypotensive episodes (Warner & Sanders, 1971; Hall *et al.*, 1972), transient cardiac arrest (Ream, 1963), and even cardiovascular collapse resulting in death (see Pittinger *et al.*, 1970) have occurred subsequent to the administration of aminoglycoside antibiotics; yet, direct cause-effect relationships were not suggested. Usually, cardiovascular changes observed under such circumstances are attributed to respiratory depression, bacteraemia, or underlying pathological conditions. In the present study, clinically normal rhesus monkeys were used to examine the myocardial and haemodynamic effects of representative aminoglycoside antibiotics in a controlled manner, and in the absence of influence by pre-existing disease. These observations establish the direct relationship of gentamicin and neomycin with suppression of cardiovascular activity in a higher primate species.

Intravenous administration of gentamicin or neomycin consistently produced a negative inotropic effect on the myocardium of healthy, surgically-prepared rhesus monkeys. Adverse cardiac contractile changes elicited by neomycin or gentamicin were invariably accompanied by a relative bradycardia and substantial decreases in systemic blood pressure and cardiac output. Similar evidence of myocardial contractile dysfunction was observed when heart rate was maintained constant by electrical pacing; therefore, tension changes produced in the heart by neomycin do not depend upon a negative chronotropic effect of this antibiotic. However, the possibility cannot be excluded that neomycin and gentamicin directly affected the vasculature causing a decrease in peripheral vascular resistance and venous return and, in this manner, contributed further to the depression of myocardial contractile strength. This possibility does not seem unlikely in view of recent evidence that aminoglycoside antibiotics inhibit vascular smooth muscle contractile function (Adams, Goodman & Weiss, 1974), and apparently have more pronounced effects on resistance arteries than on larger conduit vessels (Adams & Goodman, 1975). However, Cohen, Wechsler, Mitchell & Glick (1970) have examined the cardiac effects of streptomycin in dogs using an experimental preparation (Wildenthal, Mierzwiak, Myers & Mitchell, 1968) that maintained heart rate, aortic pressure, and cardiac output at constant levels. Under these circumstances, which circumvent chronotropic, blood pressure, and peripheral vascular influences, streptomycin produced a direct and dose-dependent suppression of myocardial contractility (Cohen *et al.*, 1970).

The mechanism of the *in vivo* negative inotropic activity of an aminoglycoside antibiotic on heart muscle was not investigated by Cohen *et al.* (1970) and, apparently, has not been examined before. Present experiments, however, provide evidence that the myocardial depressant effects of this group of agents may be associated with a modification of Ca^{2+} function. Cardiovascular parameters that had been depressed by neomycin or gentamicin in rhesus monkeys were rapidly restored to control or near control levels by Ca^{2+} administration; physiological values remained near control levels after intervention with this cation. Since Ca^{++} injection in control monkeys did not produce a sustained elevation of haemodynamic values, it seems that Ca^{2+} not only exerted a positive inotropic response in the presence of neomycin or gentamicin depression, but that this cation also completely antagonized the adverse effects of these antibiotics.

Conversely, two other positive inotropic agents, noradrenaline and isoprenaline, did not produce a sustained antagonism of the neomycin effects. These sympathomimetic amines produced rapid elevations in myocardial function in neomycin-treated monkeys. However, these positive changes were not maintained inasmuch as myocardial contractile force, dF/dt, and cardiac output returned to depressed levels within a few minutes and then gradually returned to control levels indistinguishable from the recovery values seen with neomycin alone. In control monkeys that had not been exposed to an antibiotic, however, the duration of the cardiovascular effects of noradrenaline and isoprenaline were similar to that seen with Ca^{2+}. Thus, present findings in nonhuman primates suggest that the myocardial depressant effects of aminoglycoside antibiotics may be associated with a reversible alteration of Ca^{2+} function.

Contraction of a heart cell is believed to be dependent upon the intracellular concentration of available Ca^{2+} in the vicinity of the contractile apparatus (Nayler, 1973b; Katz & Repke, 1973). Extracellular Ca^{2+} and/or Ca^{2+} that is bound to superficial areas of the sarcolemma is considered to be essential for coupling membrane excitation with intracellular events that culminate in tension changes in the myocardial cell fibre (Langer & Frank, 1972; Nayler, 1973a). Recently, the negative inotropic activity of gentamicin in isolated cardiac muscle was found to be Ca^{2+}-dependent. In electrically-driven left atria of rats, the myocardial depressant effects of gentamicin were competitively reversed by Ca^{2+}, whereas noradrenaline acted as a partial antagonist to gentamicin (Adams, 1975). These data suggested that gentamicin altered the activity of contractile-Ca^{2+} in isolated myocardium. The present *in vivo* findings may be interpreted as support for the contention that aminoglycoside antibiotics alter the activity of contractile-Ca^{2+} in the heart in some manner.

Although experimental data that directly examine the effect of an aminoglycoside antibiotic on Ca^{2+} movements in the myocardium are not available, a recent investigation has clearly shown a specific alteration of Ca^{2+} fluxes by aminoglycoside antibiotics in vascular smooth muscle. In rabbit aortic smooth muscle, gentamicin, kanamycin, streptomycin, and neomycin inhibit the uptake of radiolabelled Ca^{2+} at superficial binding sites (Adams, Goodman, Lupean & Weiss, 1973; Adams *et al.*, 1974, Goodman, Weiss & Adams, 1974). By affecting a Ca^{2+} fraction located at superficial sites, aminoglycoside antibiotics inhibit the ability of arterial muscle to respond to vasoconstrictor agents that normally use this and other interrelated Ca^{2+} pools to generate tension changes in the vessel wall (Adams *et al.*, 1974; Adams & Goodman, 1975). Aminoglycoside antibiotics may also interfere in a reversible manner with the function of Ca^{2+} in the heart cell, as they do in vascular smooth muscle. This mechanism may well explain the acute myocardial depression produced by neomycin and gentamicin in the present study and the rapidity and thoroughness of antagonism by Ca^{2+}.

The author thanks J.H. Mitchell, M.D., of the Cardiopulmonary Research Laboratory, Department of Internal Medicine, for his suggestions in the experimental design of portions of this study and for his criticism and valuable advice in the preparation of this manuscript. The technical assistance of Mr B. Mathew is gratefully acknowledged. This work was supported by contract number FDA 73-312 from the Public Health Service, Food and Drug Administration, Department of Health, Education and Welfare, U.S.A.

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(Received February 11, 1975.
Revised March 25, 1975.)

Br. J. Pharmac. (1975), 54, 463-474

VASOPRESSIN RELEASE BY NICOTINE: THE SITE OF ACTION

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1 In cats anaesthetized with chloralose the release of neurohypophysial hormones was examined after injection of nicotine into the cerebral ventricles or cisterna magna or its topical application through perspex rings to the ventral surface of the brain stem. The release was measured by assaying the hormones in samples of venous blood

2 Injected into a lateral or the third cerebral ventricle, nicotine (0.5 to 1 mg) produced release of vasopressin without oxytocin. When the aqueduct was cannulated, preventing access to the fourth ventricle and to the subarachnoid space, this release did not occur.

3 Vasopressin was also released without oxytocin when nicotine (0.25 to 2 mg) was injected into the subarachnoid space through the cisterna magna. With this route of administration the nicotine did not enter any part of the ventricular system.

4 Applied through paired perspex rings placed across the ventral surface of the brain stem, nicotine again produced release of vasopressin without oxytocin. The amount of nicotine placed in each ring was usually 80 μg, but a release was obtained with 10 μg and in one experiment with as little as 5 μg.

5 The bilateral region on the ventral surface of the brain stem where nicotine acts when producing release of vasopressin lies lateral to the pyramids and in a longitudinal direction, 6 to 9 mm caudal to the trapezoid bodies.

6 The vasopressin release by nicotine injected intraventricularly or intracisternally, or applied topically to the ventral surface of the brain stem was not due to absorption of nicotine into the blood stream, nor to blood pressure effects.

7 It is concluded that nicotine acts on the ventral surface of the brain stem probably by activating the central projection to the supra-optic and possibly also the paraventricular nuclei of afferent pathways in the sinus and vagus nerves which control the release of vasopressin in response to changes in blood volume or distribution.

Introduction

It is generally agreed that the paraventricular nuclei (PVN) are involved in the release of oxytocin, but whether these nuclei also release vasopressin is controversial. The PVN contain oxytocin and vasopressin in all species examined. Yet in the lactating guinea-pig (Tindal, Knaggs & Turvey, 1968), and rabbit (Aulsebrook & Holland, 1969), their electrical stimulation causes milk ejection without a rise in arterial blood pressure, suggesting release of oxytocin alone. On the other hand, in the cat, electrical stimulation of the PVN increases the concentration of both hormones in the blood (Bisset, Clark & Errington, 1971).

The PVN should be readily accessible to drugs injected into the cerebral ventricles, as they are contiguous to the walls of the third ventricle. The possibility was therefore considered of stimulating these nuclei by a drug injected into the cerebral ventricles in order to find out if oxytocin alone, or oxytocin with vasopressin would be released. Nicotine was chosen for this purpose. It is the drug which has been most widely used for studying the release of neurohypophysial hormones since the discovery of its antidiuretic action by Burn, Truelove & Burn (1945).

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However, when nicotine was injected into the cerebral ventricles of the anaesthetized cat, it was found to increase the concentration of vasopressin in the blood, but not of oxytocin. This unexpected finding made it doubtful that its action was on the PVN, or that it acted at all on structures lining the ventricular walls.

Cats have no foramen of Magendie, which exists only in primates, and drugs injected into the cerebral ventricles of cats pass rapidly into the subarachnoid space through the lateral recesses of the fourth ventricle and reach the ventral surface of the brain stem. Recently, it has been shown that a number of drugs which, on intraventricular injection affect blood pressure, heart rate, respiration or blood glucose, act in this way (Feldberg & Guertzenstein, 1972; Guertzenstein, 1973; Bousquet & Guertzenstein, 1973; Edery & Guertzenstein, 1974; Feldberg & Gupta, 1974; Guertzenstein & Silver, 1974; Dey, Feldberg & Wendlandt, 1975).

The following procedures are used to determine if the ventral surface of the brain stem is the site at which a drug acts on intraventricular injection. First, it is injected into a lateral or the third ventricle after cannulation of the cerebral aqueduct. If its effect is abolished the drug cannot act on structures lining these ventricles. It is then injected into the subarachnoid space through the cisterna magna. If this is effective, the likely site is either on the ventral or dorsal surface of the brain stem, since there is no access to the ventricles from the cisterna. Finally, the site can be localized by applying the drug topically through perspex rings to these surfaces. All three procedures were used in the present experiments to determine the site where nicotine acts. The technique of topical application was employed on the ventral surface only since this surface is reached more readily than the dorsal one on intraventricular injection.

Some of the results have been communicated to the Physiological Society (Bisset & Feldberg, 1972).

Methods

The experiments were done on anaesthetized male and female cats weighing between 3 and 4.5 kg. Some of the female cats were lactating. Anaesthesia was induced with ethyl chloride and ether followed by intravenous chloralose (70 mg/kg). The trachea was cannulated and the head of the cat was fixed to the ear bars and mouthpiece of a stereotaxic instrument. In the experiments in which the nicotine was injected into the cerebral ventricles or into the cisterna magna, the cat was lying on its belly; in those in which the nicotine was applied to the exposed ventral surface of the brain stem the cat was put on its back. Blood pressure was recorded from a femoral artery with a Statham strain gauge transducer and a Goertz potentiometric recorder (1 mmHg = 1.33 mbar). The same method of recording was used for determining the milk-ejection pressure from a cannulated teat duct in lactating cats in order to monitor the release of neurohypophysial hormones as described by Bisset, *et al.* (1971).

In order to prevent inhibition of the milk-ejection response by any adrenaline or noradrenaline that might be released into the circulation by nicotine, oxprenolol hydrochloride (Trasicor, Ciba) (4 mg) was injected intravenously because this β-adrenoceptor blocking agent has been shown to prevent inhibition of the milk-ejection response produced by adrenaline (Bisset, Clark & Lewis, 1967; Bisset *et al.*, 1971). Oxprenolol was given routinely also to the non-lactating cats to maintain the same experimental conditions. Atropine sulphate (4 mg) was routinely injected intravenously to prevent hypotension produced by release of acetylcholine from cholinergic neurones; in preliminary experiments it had been found not to prevent the release of vasopressin on intraventricular injection of nicotine.

The methods used for cannulating a lateral or the third cerebral ventricle with a Collison cannula were those described by Feldberg & Shaligram (1972). To cannulate the aqueduct a polythene tube of about 5 cm length was inserted through the opened cisterna along the floor of the fourth ventricle into the middle of the aqueduct so as to prevent any ventricular fluid from passing into the fourth ventricle. The outer end of the tube was bent to take up a nearly vertical position. This ensured that at least part of the nicotine solution (0.15 ml) injected into the cerebral ventricle stayed in the ventricles for some time. Injections into the cisterna magna were made as described by Feldberg & Guertzenstein (1972).

The method of exposing the ventral surface of the brain stem was that described by Feldberg & Guertzenstein (1972) except when the nicotine was applied to regions caudal to the rootlets of the XIIth cranial nerves. A diagram of the paired rings with their holder through which the nicotine was applied has been published (Guertzenstein, 1973). The rings were slightly oval, their inner diameters being 5 and 4 mm, and the distance between them 2 mm. They were placed across the medulla, each at the same distance from the midline. In order to be able to apply the nicotine to regions caudal to the rootlets of the XIIth cranial nerves, the ventral arch of the atlas as well as the dens of the epistropheus was removed with nibbling forceps and the tough dura containing a transverse venous sinus was tied on both sides as far from the midline as possible. By cutting the dura between the ligatures the ventral surface was exposed sufficiently to place the perspex rings so as to cover the regions of the rootlets of the 1st cervical nerves. The nicotine was applied in a volume of 20 µl in each ring and washed out immediately after a blood sample had been collected. The nicotine salt used was the hydrogen-tartrate but all values given in the text refer to the base. In the concentrations used the nicotine solutions were strongly acid but the vasopressin release was not due to the acidity as the release was also obtained when the solutions were neutralized. For instance, the largest vasopressin release on topical application was obtained in an experiment (No. 9 of Table 2) in which the pH of the nicotine solution was adjusted to pH 7.2.

To determine the areas on the ventral surface which were covered by the nicotine solution placed in the rings, the procedure described by Guertzenstein & Silver (1974) was adopted. The rings were filled with a 0.8% bromophenol blue solution; 5-10 min later the cat was killed by an overdose of intravenous pentobarbitone sodium. Before the rings were removed they were washed out several times with 0.9% w/v NaCl solution (saline) and the stained areas on the ventral surface were measured with a divider, using as references the caudal border of the trapezoid bodies and the mid line. The circles shown in the diagrams of Figures 3 and 4 were obtained in this way. In drawing the circles, slight variations in transverse direction were not taken into consideration but the rostral and caudal borders which were about 4 mm apart were determined within 0.5 mm.

Collection of blood samples

Samples of blood coming from the head were obtained from a cannula inserted upwards into the external jugular vein. Each sample (5 ml) was withdrawn into a nylon syringe containing a trace of heparin at a steady rate over 2 min with simultaneous replacement of warm dextran solution into the inferior vena cava through a cannulated femoral vein. In some experiments in which difficulty was experienced in withdrawing blood, the sample was taken from the femoral vein and dextran replaced through the external jugular vein.

The results obtained with the two procedures were the same.

Extraction and assay of blood samples

The samples were extracted by the method of Bisset, Hilton & Poisner (1967) which consists of precipitating the plasma proteins with alcohol and concentrating the supernatant. The extracts were assayed for vasopressin by their antidiuretic activity on intravenous injection into water-loaded rats under ethanol anaesthesia, and for oxytocin by their milk-ejecting activity on retrograde arterial injection into lactating rats treated with oxprenolol. These assay methods have been described in detail by Bisset *et al.* (1971). The standards used for the assay were synthetic arginine-vasopressin (Sandoz) and synthetic oxytocin (Syntocinon, Sandoz). The potency of the arginine-vasopressin was confirmed by assaying its antidiuretic activity against that of the III International Standard for Posterior Pituitary (Bangham & Musset, 1958).

In the assay for oxytocin, interference from the intrinsic milk-ejecting activity of vasopressin (Bisset *et al.*, 1971) was avoided by treating the extracts with trypsin before assaying them for milk-ejecting activity. Trypsin is known to inactivate vasopressin in brain extracts without affecting oxytocin (Bisset, Errington & Richards, 1973). The procedure used for blood extracts was to add 100 µg of trypsin (in 0.1 ml) to 0.5 ml extract and to incubate the mixture at 38° C for 60 min at pH 7.5. This resulted in a loss of 95% of the antidiuretic activity in the extracts. On the other hand, when a known amount of oxytocin was added to a blood sample before extraction the trypsin treatment did not reduce the recovery of its milk-ejecting activity which amounted to 95%.

In every experiment in which no oxytocin was detected in an extract, that extract was re-tested after the addition of a threshold amount of oxytocin to find out if it contained an inhibitory factor which might have masked the presence of oxytocin. The response to the added oxytocin was always found to be uninhibited.

The identity of the vasopressin in the extracts was established by showing that their antidiuretic activity was greatly reduced or abolished by treatment with trypsin, with sodium thioglycollate (Bisset *et al.*, 1971) and with a specific antibody to vasopressin (Bisset, Black, Hilton, Jones, Kanjanapothi & Montgomery, 1974).

Results

Intraventricular and intracisternal injections

As shown in Table 1, injections of 1 mg of nicotine into the cerebral ventricles caused the release of vasopressin into the blood without a release of oxytocin whether the injections were made into the third (cats 1 to 4) or into a lateral ventricle (cats 5 and 6). The vasopressin increased between 6 and 32-fold in blood samples withdrawn over a period of 2 min beginning 35 s after the injection, but in samples collected 20 min later, the vasopressin content was again low and had sometimes returned to the pre-injection level.

The collection of the blood samples was begun 35 s after the injection because in two experiments (Expts 1 and 4) on lactating cats with a teat duct cannulated, the milk-ejection pressure began to rise steeply about 35 s after the injection of 1 mg nicotine, and the rise was shown to be an effect not of released oxytocin but of released vasopressin. This is illustrated in Figure 1 which is from the same cat as Expt 1 of Table 1. The records show the effects of intravenous injections of oxytocin and of intravenous and intraventricular injections of nicotine on the milk-ejection pressure. An intravenous injection of 4 mu of oxytocin produced a large rise in pressure (at (a) in Figure 1) whereas 2 mu had a just perceptible effect (at (b)). The intraventricular injection of 0.1 mg nicotine (at (c)) had no effect on milk-ejection pressure, nor did it increase the oxytocin or vasopressin content of the blood. However, 35 s after the intraventricular injection of 1 mg nicotine (at (d)) the milk-ejection pressure began to rise steeply and the rise was of the same magnitude as that produced by 4 mu oxytocin. Yet, in the blood sample, collection of which began the moment the pressure rose, the oxytocin content was not increased, whereas its vasopressin content had risen from < 13 to 630 μu/ml. A similar result was obtained when the intraventricular injection of 1 mg nicotine was repeated after 70 min (at (e)).

Figure 1 further illustrates that absorption of nicotine into the blood stream was not responsible for the rise in milk-ejection pressure. Even if half of the 1 mg were absorbed within 35 s, although any absorption which occurs from the liquor space is far too slow for such a possibility to be contemplated, this would not have been sufficient

Table 1 Vasopressin (V) and oxytocin (O) in blood collected before (I), 35-155 s (II), and 20 min (III) after injection of nicotine into the third ventricle (cats 1-4), into a lateral ventricle (cats 5 and 6), and into the cisterna magna (cats 7 and 8)

		μu/ml of V or O					
		I		II		III	
Cat no.	*Nicotine (mg)*	*V*	*O*	*V*	*O*	*V*	*O*
1	0.02	22	<22	17	<11		
	0.1	22	<22	<13	<26		
	1			630	<13		
	1			560	<12		
2	1	<10	10-20	266	<10	15	10-20
3	1	16	10	152	<16	96	10
	1*	30	<16	660	20	216	10
4	1*	<10	<20	60	<8	<10	20
5	1	<30	<39	960	<39	97	<39
	1	<30	<39	476	<44	72	<47
	1≠	<30	<39	50	<65		
6	1	29	<48	504	<40	60	40
	1≠	42	<40	80	<80		
	1ϕ			303	<40	165	<40
7	2	25	<33	900	<33		
	0.5	24	<33	156	<33	92	<33
	2			390	<33		
8	0.5	48	<22	395	<22	48	<22
	0.25	<20	<22	216	<22	39	<22
	0.125			<20	<22		

Cats 1 and 4 lactating. * Carotid sinus denervated and vagi cut. ≠ Aqueduct cannulated. ϕ Tip of aqueductal cannula withdrawn to caudal end of fourth ventricle.

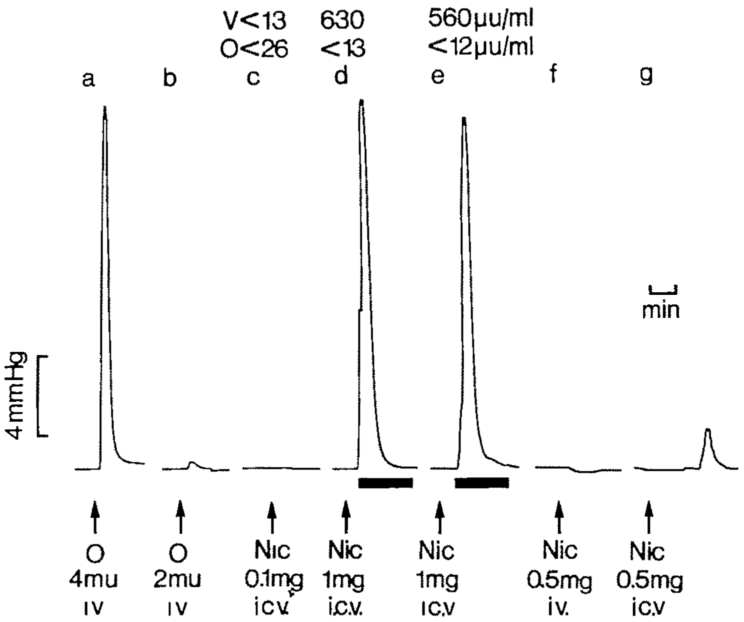

Figure 1 Records of milk-ejection pressure from a cannulated teat duct of a lactating cat anaesthetized with intravenous chloralose. Same experiment as No. 1, Table 1. Responses to intravenous (i.v.) injections of 2 and 4 mu oxytocin (O) and of 0.5 mg nicotine (Nic) and to intraventricular (i.c.v.) injections of 0.1, 0.5 and 1 mg nicotine. The figures on top give vasopressin (V) and oxytocin (O) content in μu/ml in the 2 ml samples of blood collected 35 to 155 s after the injections of 0.1 and 1 mg nicotine. The 2 min period of collection is indicated for the injections of 1 mg nicotine below the records by a solid bar.

to account for the rise in milk-ejection pressure because 0.5 mg nicotine injected intravenously (at (f)) had no effect; injected intraventricularly (at (g)), however, it was sufficient to bring about a small delayed response.

The intraventricular injection of 1 mg of nicotine produced changes in arterial blood pressure. The more usual response was a large rise in blood pressure but sometimes the response consisted mainly of a fall. It is known that a fall in blood pressure, provided it is sufficiently large and steep, causes release of vasopressin without release of oxytocin (Beleslin, Bisset, Haldar & Polak, 1967). However, the depressor effect when produced by an intraventricular injection of 1 mg of nicotine was insufficient to do so because much smaller doses of nicotine, which were ineffective in releasing vasopressin, produced larger depressor effects. This is illustrated in Figure 2 which is from the same experiment as Figure 1. Further evidence for the conclusion that the vasopressin release was independent of the fall in blood pressure was obtained in experiments in which the carotid sinuses were denervated and the vagi cut in the neck. These procedures, which are known to abolish the vasopressin release associated with the hypotension during haemorrhage (Clark & Rocha e Silva Jr, 1967) or produced by hypotensive drugs (Bisset, Kanjanapothi & Rocha e Silva Jr, unpublished experiments) did not abolish the vasopressin release produced by an intraventricular injection of 1 mg of nicotine. Experiment 4 of Table 1, shows the release produced by the nicotine injection after, and Expt 3 before and after denervating the carotid sinuses and cutting the vagi.

Two series of experiments suggested that the action of nicotine in releasing vasopressin was not on structures lining the ventricular cavities, but on structures reached after the nicotine had passed into the subarachnoid space. First, hardly any release of vasopressin occurred on intraventricular injection of 1 mg of nicotine when the aqueduct was cannulated and the passage of nicotine into the subarachnoid space was prevented. Secondly, a release occurred when the nicotine was injected into the cisterna magna, from which there is no access to the ventricles.

The effect of cannulation of the aqueduct is seen in Expts 5 and 6 of Table 1. In Expt 5, this was done after the first two intraventricular injections of 1 mg nicotine which increased the

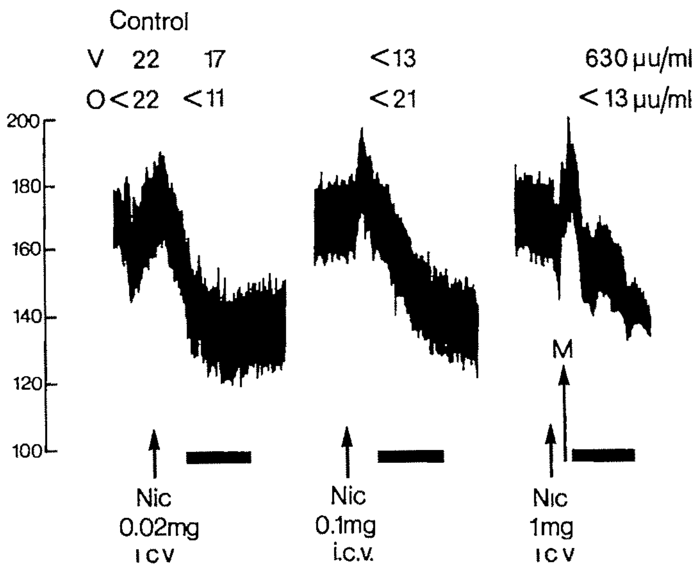

Figure 2 Arterial blood pressure from a lactating cat anaesthetized with intravenous chloralose. Same experiment as that of Figure 1. At the arrows marked Nic, intraventricular (i.c.v.) injections of 0.02, 0.1 and 1 mg nicotine. The arrow marked M indicates beginning of rise in milk-ejection pressure. The 2 min periods of collection of the blood samples are indicated below the records by solid bars. The figures on top refer to vasopressin (V) and oxytocin (O) content in µu/ml of the collected samples.

vasopressin in the blood from < 30 to 960 µu/ml after the first, and to 476 µu/ml after the second injection. The third intraventricular injection of 1 mg nicotine which was done with the aqueduct cannulated, caused a rise to 50 µu/ml only. In Expt 6, the aqueduct was cannulated after the first injection of 1 mg nicotine which had increased the vasopressin in the blood from < 48 to 504 µu/ml, but the second injection with the aqueduct cannulated caused an increase to 80 µu/ml only. The cannula was then withdrawn to the caudal end of the fourth ventricle to re-open the passage of c.s.f. through the lateral recesses and the flow into the subarachnoid space was facilitated by keeping the free end of the cannula a little above the head. In this condition, the injection of 1 mg nicotine produced again a large increase in the vasopressin content of the blood which rose to 303 µu/ml.

Both in Expts 5 and 6, the cannulation of the aqueduct had been effective in preventing the nicotine from entering the subarachnoid space. This was evident because the injection was not followed by ear twitching. Without cannulation of the aqueduct, or when the tip of the aqueductal cannula was withdrawn to the caudal end of the fourth ventricle, the injection of nicotine produced intense ear twitching. This is due to an action on structures close to the dorsal surface of the upper cervical cord which are reached by the nicotine after having entered the subarachnoid space through the lateral recesses (Armitage, Milton & Morrison, 1966; Hall & Reit, 1966). In one experiment not included in the Table, in which the tip of the aqueductal cannula did not fit the walls of the aqueduct closely and some of the injected nicotine leaked into the fourth ventricle and then passed into the subarachnoid space, ear twitching occurred and the vasopressin content of the blood rose after the injection although not as high as without cannulation.

Experiments 7 and 8 of Table 1, show the effectiveness of nicotine in releasing vasopressin on injection into the cisterna magna. Doses between 0.25 and 2 mg produced a large increase of vasopressin in the blood without any increase of oxytocin. The injections also produced intense ear twitching. As shown in Expt 8, a dose of 0.125 mg was below threshold for vasopressin release.

Topical application

The release of vasopressin without oxytocin obtained on injection of nicotine into the cerebral ventricles or into the cisterna magna was reproduced by applying nicotine bilaterally, by means of perspex rings to the caudal part of the ventral surface of the medulla oblongata. A typical experiment is illustrated in Figure 3.

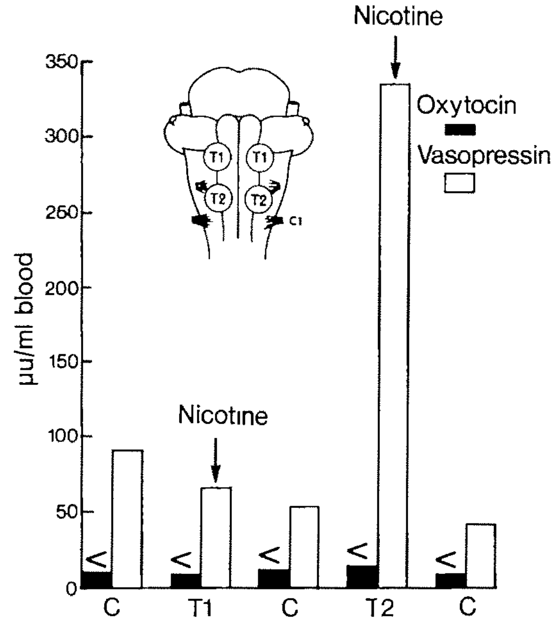

Figure 3 Oxytocin and vasopressin release on bilateral topical application of nicotine by means of perspex rings (80 μg in each ring) to two different regions of the ventral surface of the brain stem of cat. The two regions are indicated by the circles T_1 and T_2 in the diagram. C_1 rootlets of 1st cervical nerves; the rootlets above are those of the XIIth cranial nerves. Same experiment as No. 2 and No. 8 of Table 2. (For details see text.)

In this experiment, the nicotine was applied to two different regions. The regions on the ventral surface enclosed by the perspex rings during the first application of nicotine are indicated in the diagram of Figure 3 by the circles marked T_1. The regions overlap rostrally the caudal border of the trapezoid bodies by about 1 mm. The application of nicotine in the rings caused no release of oxytocin or vasopressin. A control blood sample (C) collected 20 min before the nicotine application contained < 7 μu/ml oxytocin and 89 μu/ml vasopressin and a sample, collection of which began about 35 s after placing 80 μg nicotine in each ring, contained < 6 μu/ml oxytocin and 65 μu/ml vasopressin. The nicotine was then washed out, and the rings were placed more caudally. Their new position is indicated in the diagram by the circles marked T_2; the most rostral points of the regions enclosed by the perspex rings now lay about 5 mm caudal to the trapezoid bodies and the relation of the regions to the rootlets of the XIIth cranial and the Ist cervical nerves is indicated in the diagram. From this region, nicotine produced release of vasopressin without oxytocin. Before placing the nicotine in the rings, about 1 h after collection of the second blood sample, a new control sample was collected, it contained even less vasopressin (52 μu/ml) than the first control and the oxytocin was still below threshold, < 11 μu/ml. The subsequent sample was collected a few minutes later; its collection began about 35 s after placing 80 μg nicotine in each ring. The vasopressin content of this sample increased to 334 μu/ml whereas the oxytocin content remained low, < 13 μu/ml. The nicotine was then washed out and about an hour later a further control sample of blood was withdrawn. Its vasopressin content was again low, 41 μu/ml, and its oxytocin content was < 8 μu/ml.

To determine the rostral and caudal border of the bilateral region from which vasopressin release could be obtained with nicotine, its effect was examined in different cats by placing the perspex rings at different distances caudal to the trapezoid bodies and examining the efficacy of nicotine when placing it in the rings, and sometimes also when spreading it in the same amount outside the rings either just rostral or caudal to them.

The results of 13 experiments (Nos 1-13) obtained on 12 cats are summarized in Table 2 and the regions on the ventral surface enclosed by the rings are shown by the circles in the diagram of Figure 4. No vasopressin release was obtained with nicotine placed in the rings when the rostral border of the regions enclosed by the rings either overlapped the trapezoid bodies or lay 1.5 to 2 mm caudal to them (Expts 1-4) and again when the rostral border lay 9 mm caudal to the trapezoid bodies (Expt 13). However, when the rostral border lay between 2.5 and 6 mm and the caudal border between 6.5 and 10 mm caudal to the trapezoid bodies (Expts 5-12), there was always a large release of vasopressin when the nicotine was placed in the rings. This would confine the nicotine sensitive area in longitudinal direction to between 6 and 9 mm caudal to the trapezoid bodies. This is also evident from the diagram of Figure 4. The dotted circles on the left side give the position of the rings from which no release, and the continuous circles on the right side the position of the rings from where a release of vasopressin was obtained with nicotine placed inside the rings. The same localization was obtained in those experiments in which the nicotine was spread onto the ventral surface either just caudal or rostral to the rings. In Expts 5, 7, 10 and 12, in which nicotine placed in the rings caused a release of vasopressin, no release occurred when the nicotine was spread just rostral to the rings, and in Expts 1 and 3, in which the rings were placed too far rostrally for nicotine to be

Table 2 Effect on arterial blood pressure and vasopressin content of blood, of topical application of nicotine to different regions of the ventral surface of the brain stem of cat

	Distance (mm) from trapezoid bodies of rostral (R) and caudal (C) border of regions enclosed by rings		*Changes in arterial blood pressure by nictoine in rings*	*Vasopressin content in μu/ml of blood collected before and after placing nicotine*								
				In rings			*Rostral to rings*			*Caudal to rings*		
Expt no.	*R*	*C*		*Before*	*During*	*Ratio*	*Before*	*During*	*Ratio*	*Before*	*During*	*Ratio*
1	Overlapping trap. bodies	2.0	No effect	13	13	1.0				77	199	2.7
2*	" "	3.0	Rise: 8 mmHg	89	65	0.8						
3	1.5	5.5	Rise: 10 mmHg	20	24	1.2				24	385	16.0
4	2.0	6.0	No effect	38	32	0.8						
5	2.5	6.5	Fall: 38 mmHg	23	200	8.7	18	10	0.6			
6	3.0	7.0	Fall: 90 mmHg	16	107	6.7						
7	4.5	8.5	Fall: 74 mmHg	37	248	6.7	25	25	1.0			
8*	6.0	9.0	Fall: 40 mmHg	52	334	6.4						
9	5.0	9.0	Fall: 16 mmHg	20	449	22.4						
10	6.5	9.5	Fall: 12 mmHg	24	120	5.0	27	29	1.1			
11	5.5	9.5	Fall: 52 mmHg	13	128	9.8						
12	6.0	10.0	Fall: 24 mmHg	17	230	13.5	64	62	1.0			
13	9.0	13.0	Fall: 20 mmHg	35	47	1.3	62	137	2.2	62	45	0.7
14≠	5.5	9.5		95	305	3.2						
15≠	2.5	6.5		82	178	2.2						
16≠	6.0	10.0	Fall: 50 mmHg	130	300	2.3						

The arterial blood pressure changes refer to those occurring until the end of the 2 min period of blood collection after placing nicotine inside the rings. The vasopressin content refers to blood samples collected for 2 min before and 35 to 155 s after placing 80 μg (40 μg in Expts 7 and 11) nicotine in each ring, or spreading 160 μg (80 μg in Expts 7 and 11) rostral or caudal to the rings. * Expts 2 and 8 from the same cat; same expt as Figure 3. ≠ Expts 14, 15, 16, vagi cut and carotid sinuses denervated.

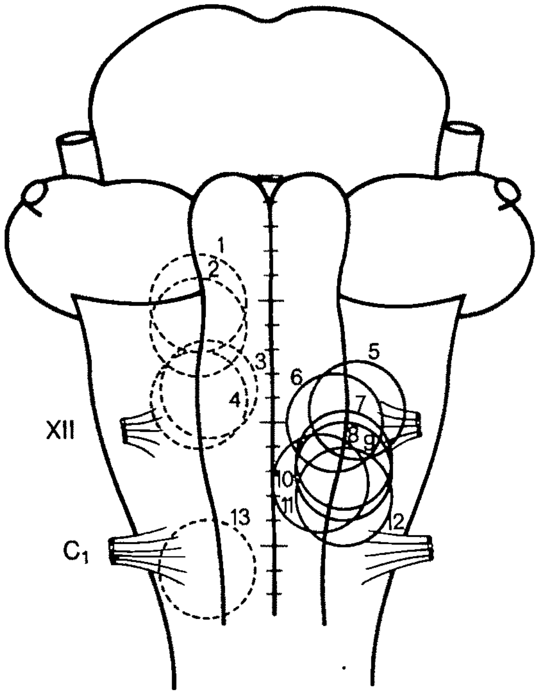

Figure 4 Diagram of ventral surface of the brain stem of the cat. The circles indicate the areas enclosed by the perspex rings in Expts 1-13 of Table 2. The number for each circle indicates the Expt No. The interrupted circles on the left refer to experiments in which no vasopressin release occurred and the full circles on the right to those in which release did occur on bilateral topical application of nicotine. C_1 rootlets of 1st cervical nerves; XII rootlets of XIIth cranial nerves. The divisions of the vertical line in the middle of the diagram correspond to 1 mm.

effective when placed in the rings, a release of vasopressin occurred when the nicotine was spread just caudal to them. Finally, in Expt 13 in which the rings were placed too far caudally for nicotine to be effective when placed in the rings, a release of vasopressin occurred when nicotine was spread rostral but not when spread caudal to them.

In none of the 13 experiments was there any detectable release of oxytocin on topical application of nicotine. For example, in Expt 9 in which the concentration of vasopressin in the blood rose to 449 μu/ml, that of oxytocin was < 14 μu/ml. This represents a vasopressin : oxytocin ratio of at least 30 : 1.

The application of nicotine to the regions from which the vasopressin release was obtained produced a fall in arterial blood pressure, whereas the application more rostrally either did not change the blood pressure, or produced a small rise. In column 4 of Table 2, the blood pressure effects are given in mmHg for 11 of the 13 experiments in which a change occurred. The values shown in this column apply only to the changes which occurred from the moment of application of nicotine in the rings to the end of the 2 min period of collection of the blood samples. The values therefore do not always give the full changes in blood pressure which took longer to develop. Although no detailed study was made of the regions from which the depressor effect was obtained its rostral border seemed to coincide with the rostral limit, but its caudal border seemed to extend beyond the caudal limit of the region from which a vasopressin release was obtained with nicotine.

The fall in arterial blood pressure cannot be the cause of the vasopressin release produced by the topical application of nicotine, although in some experiments it may have contributed to it. In Expts 5-13, the fall in blood pressure until the end of the collection of the blood samples varied between 12 and 90 mm but there was no correlation between the degree of the fall and the increase in vasopressin content of the blood. For instance, the largest release of vasopressin occurred in Expt 9 in which the fall was only 16 mm. In Expts 6, 7 and 11, the fall was 90, 74 and 52 mm respectively and could have contributed to the release. In the other experiments it was less than 40 mm and, as shown in Figure 2, a fall of this magnitude occurred after small intraventricular doses of nicotine without release of vasopressin. Moreover, in some experiments it was found that the vasopressin release did not occur with the second or third nicotine application given at hourly intervals, perhaps due to some tachyphylaxis, but the fall in blood pressure was still produced. For instance, in Expt 8, a second application of 1 mg nicotine no longer released vasopressin, but the blood pressure fell 10 mm more than during the first application which had increased the vasopressin content of the blood 6.4-fold; so it is unlikely that this vasopressin release was due to the fall in blood pressure.

In Expts 14, 15 and 16, the carotid sinuses were denervated and the vagi were cut. As this procedure often, though not always, increases the vasopressin content of the blood (Clark & Rocha e Silva, Jr, 1967) the high control values in the three experiments are readily explained. Nicotine nevertheless retained its ability to release vasopressin. With the subsequent topical application of nicotine the vasopressin in the blood rose to the same levels as in the experiments in which these nervous connections had not been interrupted. Naturally, this rise no longer meant an increase of between 5 and 13.5-fold, but of between 2.2 and 3.2-fold. The finding that denervation of the carotid sinuses and cutting the vagi did not abolish the vasopressin release produced by topical application of nicotine is further evidence for the conclusion that the release occurs independently of the blood pressure effect.

In the experiments of Table 2, the amounts of nicotine placed in each ring were 80 or 40 μg. In three experiments the effects of smaller doses were tested as well. In Expt 6, 20 μg placed in each ring did not increase the vasopressin content of the blood, but in Expt 7, this dose caused an increase from 64 to 122 μu/ml, and 10 μg caused an increase from 55 to 95 μu/ml. Finally, in Expt 10, placing 5 μg nicotine in each ring was sufficient to increase the vasopressin content of the blood from 50 to 142 μu/ml.

Discussion

The present experiments show that the release of vasopressin without oxytocin which occurs on injection of nicotine into the cerebral ventricles of anaesthetized cats was not due to an action on the paraventricular nuclei in the ventricular walls, but that the nicotine had to enter the subarachnoid space through the lateral recesses before it could act and exert its vasopressin releasing effect. This became evident when it was found that nicotine injected into the cerebral ventricles after cannulation of the aqueduct no longer produced the vasopressin release, whereas it produced such a release when injected into the cisterna magna, from which it does not enter any part of the ventricular system. This suggested the dorsal or ventral surface of the brain stem as the site of action, the ventral surface being the more likely one as it is reached more readily by the intraventricular route. This site was confirmed when the nicotine was applied bilaterally through perspex rings to the exposed ventral surface of the medulla and again produced release of vasopressin without oxytocin. These experiments, however, do not exclude the possibility of an additional site on the dorsal surface.

The amount of nicotine required to produce vasopressin release on intraventricular injection was at least 0.5 mg and usually 1 mg injected in a volume of 0.1 ml. Some dilution would occur during the passage of the drug to the ventral surface. On topical application, 160 μg were sufficient to produce a strong release, and 10 μg was the threshold dose. These doses were applied in a volume of 40 μl, i.e. in a concentration of 1/250 and 1/4000 respectively, but it has to be realised that only a fraction of the applied nicotine will penetrate the ventral surface.

By placing the perspex rings at different sites on the ventral surface, the rostral and caudal border of the nicotine-sensitive area were found to be restricted in a longitudinal direction to between 6 and 9 mm caudal to the trapezoid bodies, that is, roughly between the origins of the rootlets of the 12th cranial and 1st cervical nerves.

There was the possibility that the nicotine injected intraventricularly might reach the supraoptic nuclei from the base of the brain after having passed through the lateral recesses into the subarachnoid space and then act directly on these nuclei which are known to release vasopressin without oxytocin on electrical stimulation. This possibility does not apply to the release on topical application because the nicotine-sensitive area is strictly circumscribed and remote from these nuclei. We must therefore assume that nicotine applied topically, and probably also when injected intraventricularly, does not act on the neurosecretory cells directly, but is stimulating them reflexly.

The afferent pathway of the milk-ejection reflex from the mammary glands to the oxytocin releasing cells of the paraventricular nuclei has been worked out in detail and defined almost in its entirety (Cowie & Tindal, 1971). Much less is known about the reflex which controls the release of vasopressin in response to changes in blood volume and distribution (for reviews see Bisset, 1975; Bisset & Jones, 1975). The afferent pathway of this reflex consists of fibres arising from baro- and stretch receptors which enter the brain stem in the sinus and vagus nerves. If the nicotine acts on this pathway the present experiments suggest a nicotine-sensitive synapse between the afferent fibres and their central projection to the neurosecretory cells which release vasopressin from the supra-optic and possibly also the paraventricular nuclei. However, since the sinus and vagus nerves enter the brain stem rostral to the region on which nicotine acts the afferent fibres would have to turn caudally for a short distance to reach the synapse or form a connection with it through one or more internuncial neurones. Such a synapse close to the ventral surface of the brain stem would perhaps be accessible to electrical stimulation and lesions. In this way the results obtained with nicotine may help to elucidate in more detail the neural control of vasopressin release.

The fall in arterial blood pressure which occurs on topical application of nicotine is not the cause of the vasopressin release. Not only was there no correlation between the two effects, but the vasopressin release was also obtained after the sinus and vagus nerves were cut. A fall in arterial blood pressure produced by nicotine in anaesthetized cats, and attributed to an action on structures at the ventral surface of the brain stem had been described earlier by Armitage & Hall (1967). They obtained a strong depressor effect when nicotine was perfused from the fossa interpeduncularis to the cisterna magna.

As the bilateral area from which vasopressin release was obtained with nicotine lies 6-9 mm caudal to the trapezoid bodies the area is situated more caudally on the ventral surface of the medulla than the bilateral area from where most of the blood pressure effects have been obtained with drugs topically applied through perspex rings (Feldberg & Guertzenstein, 1972; Guertzenstein, 1973; Bousquet & Guertzenstein, 1973; Edery & Guertzenstein, 1974; Guertzenstein & Silver, 1974). For pentobarbitone sodium, glycine and clonidine, it was shown that they no longer produced their depressor effects with the rings placed so that their rostral border was 5 mm caudal from the trapezoid bodies, and the glycine-sensitive area was restricted in longitudinal direction to between 1 and 2.5 mm caudal to the trapezoid bodies. Nicotine was shown in the present experiments to produce depressor effects from more caudally situated regions. Cholinomimetic substances seem to be effective also from regions extending more caudally than the pentobarbitone sodium-, glycine-, and clonidine-sensitive area. Loeschcke and his co-workers distinguished on the ventral surface lateral to the pyramid, three chemosensitive zones from which respiratory effects, and sometimes blood pressure effects as well, were obtained on electrical stimulation and on topical application of drugs or solutions with an abnormal pH. The three areas were given the letters M, S and L, standing for Mitchell, Schlaefke and Loeschcke (Trouth, Loeschcke & Berndt, 1973a). The area M lies on the trapezoid bodies. The area S lies about 2.5 to 5 mm caudal to the trapezoid bodies. It corresponds to an area in which Petrovický (1968) found groups of nerve cells immediately under the pia mater within the marginal glia, i.e. in close proximity to the liquor. Both areas are situated more rostrally than the area from which vasopressin release was obtained. The area L on the other hand, lies 5.5 to 9 mm distal to the trapezoid bodies and corresponds to the region from where vasopressin release was obtained. According to Trouth *et al.* (1973b), this area contains groups of large multipolar ganglion cells close to the ventral surface which could be the morphological substrate for its 'chemosensitivity' including that for nicotine.

P.G.G. and M.R.E.S. Jr. were Wellcome Research Fellows. We should like to thank Miss Margaret C. Montgomery for her help with the bioassay.

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Br. J. Pharmac. (1975), 54, 475-479

HYPOTHERMIC EFFECT OF SODIUM ACETYLSALICYLATE ON AFEBRILE MONKEYS

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1 Afebrile monkeys (*Macaca cyclopis*) receiving systemic (100-300 mg/kg, i.p.) or central (5-20 mg into the 3rd cerebral ventricle) administration of sodium acetylsalicylate showed a dose-dependent reduction in rectal temperature in a thermoneutral environment (25° C).

2 Administration of sodium acetylsalicylate (10 mg) into the 3rd cerebral ventricle produced a hypothermia with a temperature decrement of 1.0° C, while an intraperitoneal injection of 300 mg/kg was required for a temperature decrement of 0.9° C. The ratio between the total doses given by the two routes was 1 to 120.

3 Following the administration of sodium acetylsalicylate, a decline in rectal temperature was accompanied by a tail cutaneous vasodilatation.

4 The data suggest that sodium acetylsalicylate can lower the normal body temperature by activating heat loss or decreasing the normal (tonic) inhibition of the heat loss mechanism via the central nervous system.

Introduction

It is generally agreed that salicylates produce an antipyretic effect in animals (Chai, Lin, Chen & Wang, 1971; Cranston & Rawlins, 1971; Lin & Chai, 1972; Rawlins, 1973) and humans (Rosendorff & Cranston, 1968) made febrile with pyrogen. Previous studies have also indicated that both pyrogen and salicylate act in the same central thermoregulating structures (most probably the hypothalamus); pyrogen by inhibition of the heat loss mechanism and salicylates by release of such inhibition (Wit & Wang, 1968; Luff, Rawlins & Wright, 1971; Lin & Chai, 1972). Our recent results have also demonstrated that salicylates lower hyperthermia during external heat exposure by activating the heat loss mechanism (Lin & Chai, 1975).

In afebrile animals, however, the effect of salicylates on body temperature is less clear. No temperature change has been observed in afebrile human subjects (Rosendorff & Cranston, 1968) or rabbits (Cranston, Luff, Rawlins & Rosendorff, 1970). In contrast, rats receiving systemic injections of salicylates showed a severe reduction in normal body temperature when placed in a cold environment (Satinoff, 1972; Franscesconi & Mager, 1974). No work has been reported so far in monkeys. Furthermore, little is known as to whether the direct injection of salicylates into the cerebral ventricle will affect normal body temperature.

Thus, the present investigation was carried out with conscious afebrile monkeys in which the thermosteady state was first reached by adapting the animals to a thermoneutral environment (25° C). Sodium salicylate was subsequently administered by different routes and in various doses.

Methods

Fourteen monkeys (*Macaca cyclopis*) of either sex weighing 3.5 to 4.5 kg were used in the present study. Each animal was anaesthetized with sodium pentobarbitone, 30 mg/kg, intravenously. A 22-gauge cannula was surgically implanted into the 3rd cerebral ventricle of each animal with the aid of a stereotaxic instrument for drug administration at a later time. This technique has been described by Chai *et al.* (1971) and Hoo, Lin, Wei, Chai & Wang (1972). At the end of the experiments, the animals were killed and the heads were perfused with 10% formalin-saline. Location of the cannula was verified by gross inspection and histological section with thionin stain. Intraperitoneal injections were given by direct puncture of the abdominal wall.

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Each animal was allowed at least one week to recover completely from the surgery before being subjected to experiments. Before drug administration, the animal was seated quietly in a primate chair at an ambient temperature of 25 ± 0.5° C until it maintained a steady body temperature for at least 1 hour. During the control period of observation, any animals showing a body temperature below 37.5° C or above 39.0° C were not used. Successive drug administrations were at least one week apart to eliminate any possible tolerance effect. The evening before the experiment, each animal was given water *ad libitum*, but food was withheld. The rectal temperature was monitored by means of a Tri-R-6 flexible thermistor probe and the subcutaneous temperature of the tail was monitored by a needle thermistor. All the recordings were made continuously on a Grass polygraph.

Sodium acetylsalicylate (0.5 to 2.0 g) was freshly dissolved in 10 ml of distilled water, adjusted to pH 7.0-7.2, to make 0.25 to 1.0 M solutions. A volume of 0.1 ml of this solution, containing 5 to 20 mg of sodium acetylsalicylate, was injected into the 3rd cerebral ventricle (intracerebroventricular; i.c.v.). Sodium chloride solutions of equal concentration were prepared as controls to be injected in similar volumes via the same route. For intraperitoneal injection, sodium acetylsalicylate was dissolved in a 0.15 M NaCl solution to make several concentrations ranging from 100 to 300 mg/ml.

Results

At a thermoneutral environment (25° C), monkeys treated with sodium acetylsalicylate intraperitoneally or intracerebroventricularly, showed a significant fall in their rectal temperature when compared with animals treated with saline (Figures 1 & 2).

Intraperitoneal administration of sodium acetylsalicylate

Figure 1 shows that sodium acetylsalicylate (100-300 mg/kg, i.p.) induced a dose-dependent hypothermic effect in eight afebrile animals. Each animal served as its own control. With a dose of 100 mg/kg, the rectal temperature began to decrease 35 ± 4.8 min after injection and returned to its control level at 120 ± 12.6 minutes. The maximum decrement in rectal temperature was 0.2 ± 0.12° C (38.3-38.1° C) which was not significant ($P > 0.2$). When the dose was increased to 200 mg/kg, the time to the onset of hypothermia was reduced (12 ± 3.1 min), the maximum decrement in rectal temperature was augmented (0.6 ± 0.09° C; 38.2-37.5° C; $P < 0.05$), and the recovery time was prolonged (180 ± 20.5 minutes). After the injection of 300 mg/kg, the maximum decrement in rectal temperature was further augmented (0.9 ± 0.11° C; 38.1-37.2° C; $P < 0.01$) with a shorter latency of onset (3.5 min) and a longer period of recovery (240 ± 14.5 minutes).

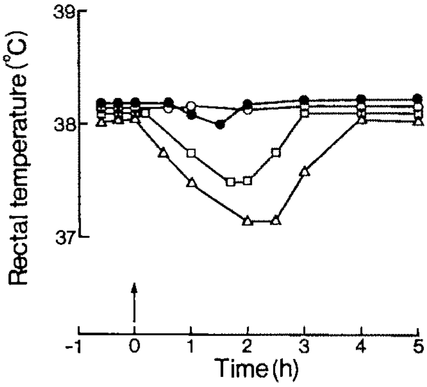

Figure 1 Change in rectal temperature of conscious, afebrile monkeys kept at an ambient temperature of 25° C for 5 h after intraperitoneal administration of sodium acetylsalicylate at arrow. Each point is the average temperature of 8 animals. Control intraperitoneal injection of saline (○); after intraperitoneal injection of sodium acetylsalicylate, 100 mg/kg (●), 200 mg/kg (□) and 300 mg/kg (△).

Intracerebroventricular administration of sodium acetylsalicylate

Similarly, Figure 2 shows that injection of sodium acetylsalicylate into the 3rd cerebral ventricle induced a dose-dependent hypothermia in six different afebrile animals. Again, each animal served as its own control. An intracerebroventricular dose of 5 mg of sodium acetylsalicylate produced a slight fall of 0.5 ± 0.12° C (38.3-37.8° C; $P < 0.05$) in rectal temperature. The time to onset of hypothermia was 30 ± 4.2 min and the recovery time was 160 ± 11.6 minutes. When the dose was increased to 10 mg, the time to onset of hypothermia became shorter (4 ± 2.1 min), the maximum decrement in rectal temperature greater (1.0 ± 0.22° C; 38.3-37.3° C; $P < 0.05$), and the recovery time longer (240 ± 16.2 minutes). This is comparable to the hypothermic effect obtained by

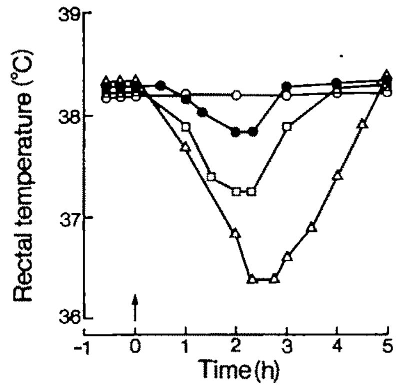

Figure 2 Change in rectal temperature of conscious, afebrile monkeys kept at an ambient temperature of 25° C for 5 h after injection of sodium acetylsalicylate into the 3rd cerebral ventricle at arrow. Each point is the average temperature of 6 animals. Control injection of saline (○); after sodium acetylsalicylate, 5 mg (●), 10 mg (□) and 20 mg (△).

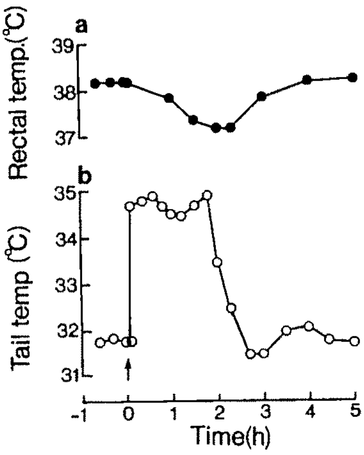

Figure 3 The general pattern of temperature changes of monkeys receiving 10 mg of sodium acetylsalicylate via 3rd cerebral ventricle at arrow. Each point is the average temperature of 6 animals. (a) rectal temperature; (b) tail cutaneous temperature.

the intraperitoneal administration of 300 mg/kg. With a dose of 20 mg of sodium acetylsalicylate, the maximum decrement in rectal temperature was further augmented to 2.0 ± 0.36° C (38.37-36.37° C; $P < 0.01$). The rectal temperature fell almost immediately after injection and returned to the control level much later (300 ± 21.4 minutes).

Figure 3 shows the general pattern of rectal and tail cutaneous temperature changes in response to intracerebroventricular acetylsalicylate. It was found that, concomitant with the onset of the falling rectal temperature, the tail cutaneous temperature increased greatly and rapidly. The tail cutaneous temperature remained at a high level (2-3° C above control) during the decreasing phase of rectal temperature, but then fell rapidly 10-15 min before the hypothermia reached its maximum.

Discussion

The present investigation was carried out in normothermic afebrile monkeys. The afebrile state was carefully checked before each experiment and it was found that, during the control period of observation, the mean rectal temperature of these monkeys was 38.2° C (ranging between 38.1 and 38.4° C). This coincides with the earlier reports of other investigators (Eyre & Kennedy, 1907; Funkhouser, Higgins, Adams & Snow, 1967; Nakayama, Hori, Nagasaka, Tokura & Tadaki, 1971).

Findings from the present experiments are that afebrile monkeys receiving sodium acetylsalicylate (100-300 mg/kg, i.p.) produce a dose-dependent decline in rectal temperature (−0.2 to −0.9° C) when placed in a thermoneutral environment (25° C). These results are consistent with recent reports of other investigators who demonstrated, in addition, that rats treated with salicylates (30-600 mg/kg, i.p.) displayed a more severe reduction in rectal temperature when placed in a 4-5° C environment (Satinoff, 1972; Franscesconi & Mager, 1974). The present results also have shown that the injection of sodium acetylsalicylate (10 mg) into the 3rd cerebral ventricle produced the same hypothermic effect as an intraperitoneal dose of 300 mg/kg (or 1200 mg for a monkey weighing 4 kg). The ratio between these two doses is 1 to 120. This suggests a central site of action. Furthermore, the present study has demonstrated that a decline in rectal temperature, following the administration of sodium acetylsalicylate, was accompanied by a persistent cutaneous vasodilatation. Therefore, these findings suggest that sodium acetylsalicylate lowers the normal body temperature by activating heat loss or decreasing the normal (tonic) inhibition of the heat loss mechanism in the central nervous system.

Similarly, it has been shown that the antipyretic effect of sodium acetylsalicylate is of central origin (Chai *et al.*, 1971). An intracerebroventricular (4 mg) or an intravenous (100 mg/kg) dose of the drug produced a marked antipyresis in monkeys with fever induced by leukocytic pyrogen. However, in afebrile monkeys of the present study, an intraventricular dose of 10 mg or an intraperitoneal dose of 200 mg/kg was required to achieve a marked decline in rectal temperature. Thus, it seems that the febrile state is more sensitive to the administration of sodium acetylsalicylate. Indeed, it has been observed that, following an intravenous administration of [^{14}C]-acetylsalicylate, the concentration of the drug in the brain of febrile rabbits is significantly higher than that in afebrile animals (Chai, Lin, Hoo & Wang, unpublished data). It is not impossible that the elevated body temperature with high metabolic rate increases the entrance of sodium acetylsalicylate into the brain and thus enhances the heat dissipating mechanism.

On the other hand, the present results seem at variance with those reports that have demonstrated that salicylates have little or no effect on body temperature of afebrile rabbits (Cranston *et al.*, 1970) and humans (Rosendorff & Cranston, 1968). Cranston *et al.* (1970) have shown that salicylates (60 mg/kg i.v. or 1.2 mg i.c.v.) had no significant effect on rectal temperature. Similarly, in human volunteers, salicylates (2 g or 30 mg/kg, i.v.) also had no effect on normal body temperature (Rosendorff & Cranston, 1968). In fact, the present study has clearly demonstrated that sodium acetylsalicylate in low doses (i.e. 100 mg/kg, i.p. or less than 5 mg, i.c.v.) failed to induce hypothermia. Therefore, it seems justifiable to explain the difference between these two sets of results as a difference in the dosage of salicylates being used.

In unanaesthetized monkeys, Myers & Yaksh (1971) found that perfusion of the cerebral ventricles with artificial c.s.f. caused hypothermia when the solution contained 24-48 mM excess calcium ion and hypothermia when 11-34 mM excess sodium ion was perfused. In the present study, a single dose of 0.1 ml containing 0.25-1.0 M sodium acetylsalicylate was administered into the 3rd ventricle. It is unlikely that the sodium or calcium concentrations in the c.s.f. were sufficiently disturbed by our injections to produce hypothermia.

More recently, Feldberg, Gupta, Milton & Wendlandt (1973) have demonstrated that the prostaglandin-like activity of c.s.f. increased greatly during bacterial pyrogen fever, and decreased when the temperature was brought down by salicylates. In addition, Franscesconi & Mager (1974) have shown that the brain levels of tryptophan and hypothalamic 5-hydroxytryptamine were elevated in rats with salicylate-induced hypothermia. Whether these substances play any role in the salicylate-induced hypothermia in afebrile animals remains to be determined.

This work was supported by grants from the J. Aron Charitable Foundation (U.S.A.) and National Science Council (Republic of China). The authors are grateful to Dr C.T. Loo (for encouragement and support) and Miss Lea Nordlicht (for preparation of this manuscript). M.T.L. is a recipient of an NIH International Postdoctoral Research Fellowship: PHS1 FO5 TW2081.

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(Received November 11, 1974.
Revised April 22, 1975.)

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THE EFFECTS OF LYSERGIC ACID DIETHYLAMIDE ON THE RESPONSE TO FIELD STIMULATION OF THE RAT VAS DEFERENS AND THE RAT AND CAT ANOCOCCYGEUS MUSCLES

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1 The effect of lysergic acid diethylamide (LSD) on the response to field stimulation *in vitro* of the rat vas deferens and anococcygeus muscle was examined.

2 LSD in concentrations from 10^{-9} to 10^{-6} M caused an increase in tone or rhythmic activity in both tissues, effects identical to those produced by guanethidine or tyramine. The motor effects of all three drugs were abolished by phentolamine 2×10^{-6} M. Methysergide 2×10^{-7} M given before LSD reduced the motor effect but was ineffective once the LSD contraction had developed.

3 LSD 10^{-9} to 10^{-6} M reduced and eventually abolished the response to motor adrenergic nerve stimulation in the anococcygeus muscle with no effect on the response to noradrenaline (NA) and no evidence of differential sensitivity according to the number of stimulating pulses. In the vas deferens LSD abolished the initial twitch component with no effect on the secondary slow contraction. LSD had no effect on the response to inhibitory nerve stimulation in the anococcygeus.

4 These results suggest that in the anococcygeus LSD closely resembles guanethidine in its effects as an adrenergic neurone blocking drug with indirect sympathomimetic actions. In the vas deferens these properties would explain the block of the initial twitch component in the motor response to field stimulation and the increase in rhythmic activity but do not explain the resistance of the secondary slow component of the motor response.

Introduction

Lysergic acid diethylamide (LSD) has been shown to produce at least two effects on smooth muscle preparations. First, it has been reported to cause contraction in the cat nictitating membrane (Thomson, 1958), the sheep umbilical artery (Dyer & Gant, 1973) and the rat and cat anococcygeus muscles (Gillespie & McGrath, 1974a). Secondly, LSD can inhibit the motor response to field stimulation of nerves in the rat and guinea-pig vas deferens (Ambache, Dunk, Verney & Aboo Zar, 1973) and recently this observation has been extended to the rat anococcygeus muscle (Ambache, Killick, Srinivason & Aboo Zar, 1973).

The pharmacology of motor transmission in the vas deferens presents several unusual features which have prompted Ambache & Aboo Zar, 1971 to suggest that the transmitter is not noradrenaline (NA). In contrast all of the evidence obtained in our laboratory suggests that the motor nerves in the anococcygeus are conventionally adrenergic. We have, therefore, re-examined the effect of LSD on the rat vas deferens and compared this with the effect on the rat and cat anococcygeus muscles to try to determine whether the action of LSD in both organs is consistent with a motor adrenergic innervation. The findings indicate that LSD is both an indirect sympathomimetic and an adrenergic neurone blocking drug and that these two effects explain all of its actions on the anococcygeus. In the vas deferens, LSD also possesses these properties and this explains some but not all of its actions on this tissue. A preliminary report of some of these findings has been published (Gillespie & McGrath, 1974b).

Methods

Two types of preparation were used. Most work was on the isolated vas deferens or anococcygeus but in addition some *in vivo* experiments were

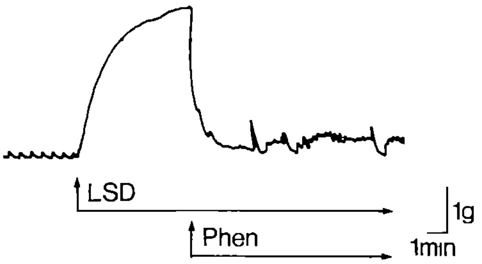

Figure 1 The motor response of the rat anococcygeus to lysergic acid diethylamide (LSD, 10^{-6} M) and its abolition by phentolamine (Phen, 10^{-6} M).

done on the pithed rat with a moveable spinal electrode to allow selective stimulation of spinal nerve outflows.

The isolation of the rat and cat anococcygeus muscles has already been described (Gillespie, 1972, Gillespie & McGrath, 1974a). Since the rat muscles are heavier and stronger in the male, only male animals were used but the cats were of either sex. The muscles were mounted in ring electrodes in a 10 ml bath containing Krebs-Henseleit solution at 37° C and gassed with a mixture of 95% O_2 and 5% CO_2. Tension was measured with a Grass FTO3 isometric transducer and displayed on a Grass Polygraph. The rat vas deferens preparations were set up in a similar manner with the stimulating electrodes around the prostatic end of the muscle where the motor nerves enter the tissue. Field stimulation of the intramural nerves in these isolated tissues was with 1 ms pulses at supramaximal voltage at the frequencies indicated in the text.

In some preparations the terminal adrenergic nerve plexus was destroyed by exposure to relatively high concentrations of 6-hydroxydopamine (6-OHDA) added to the bath as described by Wadsworth (1973). The bath concentration of 6-OHDA used was 200 μg/ml; the drug was freshly prepared immediately before addition to the bath and it was left in contact with the tissue for 2 hours. The 6-OHDA was not protected by ascorbic acid since this itself was found to cause contraction of the anococcygeus muscle. The efficacy of this technique was shown by its ability to abolish the effect of motor nerve stimulation and of the indirect sympathomimetic, tyramine, with no reduction in the response to NA and also by the disappearance of fluorescent nerve fibres when the tissue was examined with the formaldehyde fluorescence technique of Falck & Hillarp.

The method of preparation of the pithed rat has previously been described (Gillespie, MacLaren & Pollock, 1970). The moveable spinal electrode was placed to stimulate the nerve outflows between L1-L3 and again 1 ms pulses at supramaximal voltages were used. The method of recording tension from the anococcygeus muscles or vas deferens has also been described (Gillespie & McGrath, 1973). Systemic blood pressure was recorded from one femoral artery and drugs administered via a cannulated femoral vein.

The following drugs were used and concentrations are expressed as molar in the experiments *in vitro* and in grams of the base in the experiments in the pithed rat: guanethidine sulphate (Ciba), 6-hydroxydopamine hydrochloride (Calbiochem), lysergic acid diethylamide (Sandoz), methysergide bimaleate (Sandoz), noradrenaline bitartrate (Koch-Light), phentolamine mesylate (Ciba), tyramine hydrochloride (Sigma).

Results

Agonist action of LSD

In both the anococcygeus and the vas deferens, LSD in concentrations between 10^{-9} and 10^{-6} M caused contraction (Figures 1 and 2). In the anococcygeus the response was large, developed slowly in comparison with the effect of NA and was slow to reverse on washing the drug out of the bath. Repeated exposure to the drug resulted in a diminution in the response with no change in the response to NA. This tachyphylaxis also affected the indirect sympathomimetic action of tyramine and guanethidine. In the vas deferens LSD's ability to cause contraction was less dramatic and if the sensitivity of the recorder was appropriate to the maximum contractions induced by motor nerve stimulation then the motor effect of LSD could be overlooked. If however, the sensitivity was increased the LSD was shown in every preparation to cause a prolonged period of spontaneous mechanical activity (Figure 3). This pattern of activity was identical with that induced by two other indirect synpathomimetics, tyramine and guanethidine, as shown for guanethidine in Figure 2.

This motor effect in both the anococcygeus and the vas appeared to be due to an action on smooth muscle α-adrenoceptors since in both tissues it was abolished by phentolamine 2 x 10^{-6} M (Figures 1 and 2). Such an action could have been due either to a direct agonist interaction between LSD itself and the α-receptors or to an indirect sympathomimetic action of LSD and interaction of the released NA and the α-receptors. These alternatives were tested by examining the action of LSD on tissues previously exposed to 6-OHDA to destroy

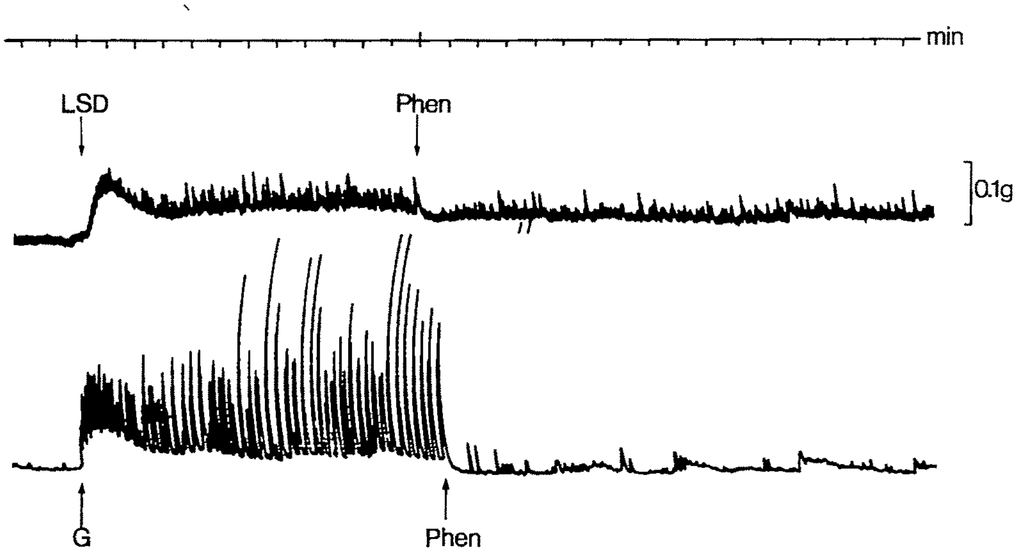

Figure 2 The motor effect of lysergic acid diethylamide (LSD, 10^{-7} M) and of guanethidine (G, 3×10^{-5} M) on the rat vas deferens and its abolition by phentolamine (Phen, 10^{-6} M). The rise in tone and increase in rhythmic activity is small and is clearly seen only if the recording sensitivity is high.

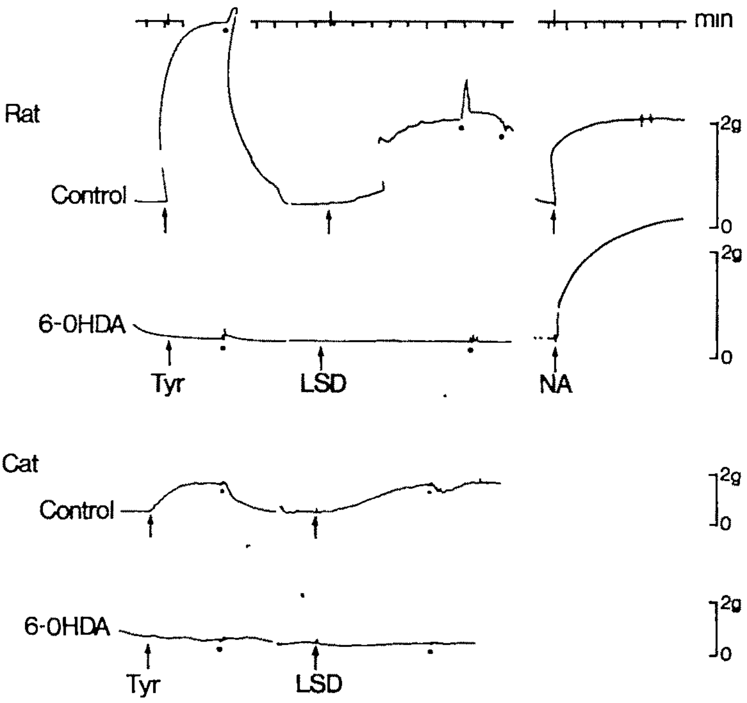

Figure 3 The effect of treatment with 6-hydroxydopamine (6-OHDA) on the response of the rat (upper two records) and the cat (lower two records) anococcygeus muscles to lysergic acid diethylamide (LSD, 2×10^{-8} M) and tyramine (Tyr, 10^{-5} M) and of the rat muscle to noradrenaline (NA, 10^{-7} M). Treatment with 6-OHDA abolished the motor response to both tyramine and LSD but potentiated the response to NA.

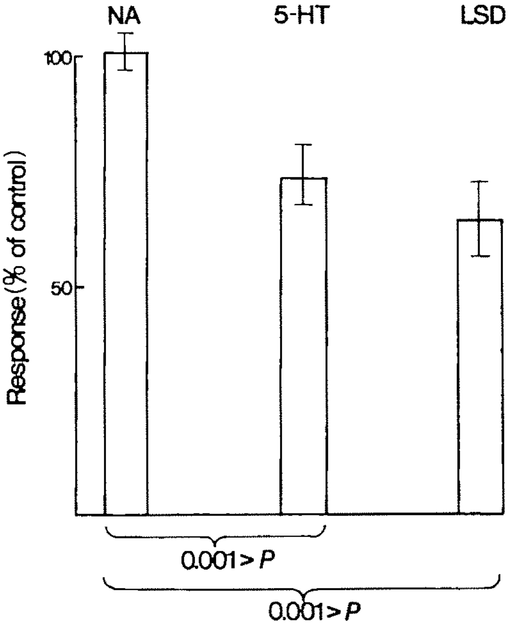

Figure 4 The effect of methysergide (2×10^{-7} M) on the motor response of the rat anococcygeus to noradrenaline (NA, 10^{-5} M), 5-hydroxytryptamine (5-HT, 10^{-5} M) and lysergic acid diethylamide (LSD, 10^{-7} M). The assessment of statistical significance shown below is by Student's *t* test.

the adrenergic nerves. In both the anococcygeus and the vas deferens motor effects in response to LSD, tyramine or guanethidine could not be obtained after treatment with 6-OHDA although the response to NA was still present and indeed enhanced. These results for the cat and rat anococcygeus are illustrated in Figure 3.

LSD is more commonly associated with the receptors for 5-hydroxytryptamine (5-HT) and for this reason we examined the effect of methysergide (2×10^{-7} M) on the motor effect of LSD. Unlike phentolamine, methysergide was found to be quite ineffective in abolishing the response to LSD once this was established. Methysergide, however, if added before LSD did prevent the development of contraction. The development of tachyphylaxis made this kind of experiment difficult to interpret using the control response to LSD in the same muscle. We, therefore, used paired muscles from the same animal mounted in separate baths, taking as control the response to LSD alone in one muscle and comparing this with the response to LSD in the presence of methysergide in the paired muscle. This method had the advantage that only one dose of LSD was used in each muscle but the disadvantage that the variability in responses was increased. The effects of methysergide with this technique on the responses to LSD (10^{-7} M), 5-HT (10^{-5} M) and NA (10^{-5} M) on the rat anococcygeus are shown in Figure 4. The responses to NA were unaltered (101 ± 4% control) while those to LSD (65 ± 8% control) and to 5-HT (74 ± 7% of control) were inhibited. Methysergide itself in concentrations up to 10^{-5} M did not cause contraction in any of the tissues tested.

In the pithed rat intravenous injection of LSD 200 μg/kg caused contraction of both the anococcygeus and the vas deferens.

Effect of LSD on the responses to motor nerve stimulation

In the experiments described by Ambache *et al.*, 1973a & b, short trains of pulses were used to cause contraction and in the vas deferens in particular there is evidence of a differential sensitivity of the responses elicited by long and short trains of stimuli to pharmacological agents. For this reason we have examined the effect of LSD on the response to trains of different length varying from 1 to 100 stimuli but at a fixed frequency of 10 Hz. In the anococcygeus, LSD concentrations from 10^{-9} to 10^{-6} M inhibited the motor response to field stimulation in a manner identical to that of guanethidine though effective at lower concentrations than the latter drug (Figure 5). Unlike guanethidine all concentrations of LSD which reduced the response to nerve stimulation also caused contraction so that LSD commonly raised tone and simultaneously reversed the response to field stimulation to inhibition. This effect complicated the measurement of the reduction in the motor nerve response. This problem was overcome by increasing gradually the dose of LSD so that tachyphylaxis of its motor effect minimized the rise in base line. In this way the inhibitory effect of increasing doses of LSD on the motor nerve response was measured and the results are illustrated in Figures 6 and 7 for the rat anococcygeus and vas deferens. In the anococcygeus LSD almost completely inhibited the motor response and there was no evidence of a preferential effect on the response with short trains of stimuli.

The motor response of the vas deferens to field stimulation was more complex. The full response was seen with trains of stimuli greater than 20 pulses and consisted of an initial rapid contraction or 'twitch' followed by partial relaxation and then a secondary slower development of a maintained tension (Figure 6). Shorter trains of pulses favoured the twitch component of the response so that up to about 10 pulses there was no evidence

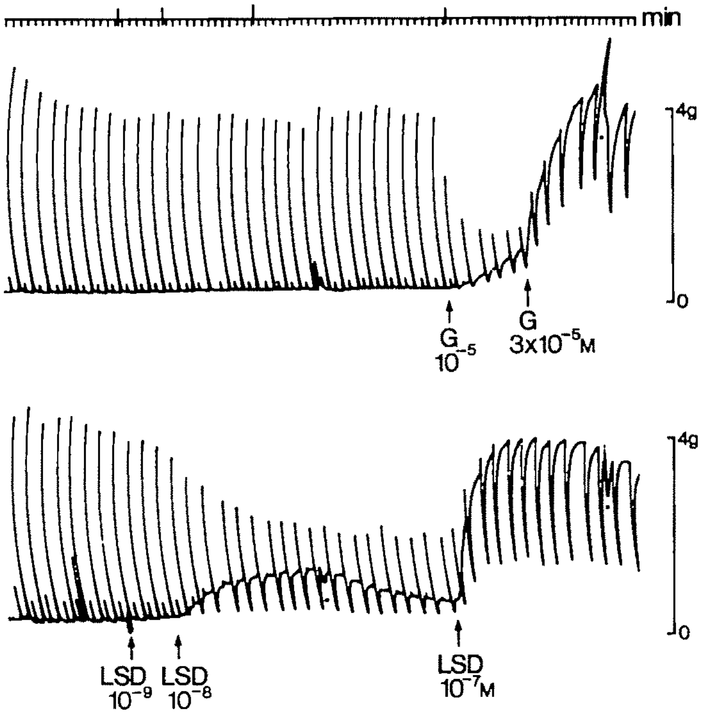

Figure 5 The effect of guanethidine (G) and lysergic acid diethylamide (LSD) on the response of the cat anococcygeus muscle to field stimulation. The nerves were alternatively stimulated with either a single stimulus or trains of 30 stimuli at 10 Hz. Both LSD and guanethidine inhibited the motor response, raised tone and reversed the response to field stimulation to inhibition.

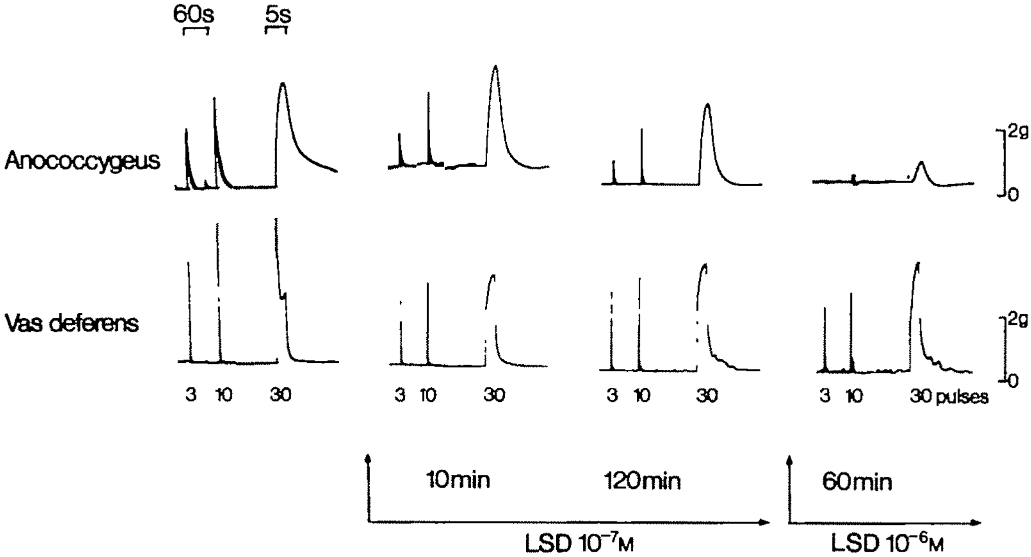

Figure 6 The effect of lysergic acid diethylamide (LSD) on the response of the rat anococcygeus muscle (upper records) and vas deferens (lower records) to stimulation with 3, 10 or 30 pulses at 10 Hz. In the anococcygeus the motor response is progressively inhibited until it is almost abolished. In the vas the control motor responses at 3 and 10 pulses consist of a single twitch but at 30 pulses the second slow component appears. LSD selectively inhibits the initial twitch component but potentiates the secondary response.

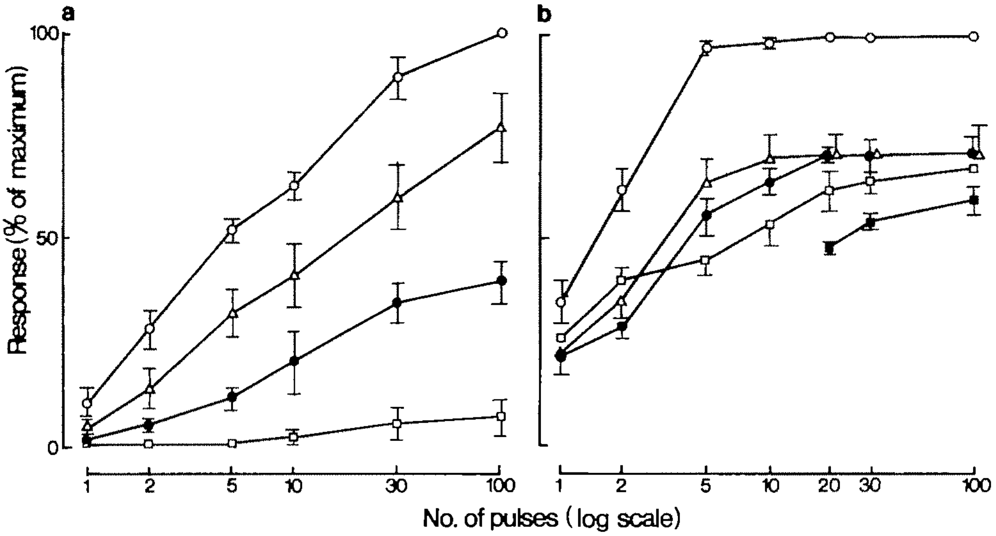

Figure 7 The effect of lysergic acid diethylamide (LSD) on the motor response of (a) the anococcygeus muscle and (b) the vas deferens to field stimulation at 10 Hz. The number of pulses is shown on the abscissae and the response as a percentage of the maximum on the ordinates. In the vas the amplitude of the initial spike response (○) and the secondary slow response (■) were measured in the control, in the anococcygeus there is only one component to the control response (○). The remaining graphs show the response in the presence of LSD 10^{-8} M (△), 10^{-7} M (●) and 10^{-6} M (□).

of a secondary component; with longer trains the amplitude of this secondary component increased though it never exceeded about 80% of the twitch response. LSD in concentrations from 10^{-9} to 10^{-6} M preferentially inhibited the initial twitch response with no effect on the secondary component or sometimes potentiating this (Figure 6). This effect of LSD was easy to demonstrate qualitatively but difficult to measure quantitatively since reduction of the 'twitch' caused it to merge with the secondary component and prevent further assessment. In Figure 7 we have separately measured, in control, the height of the twitch and of the secondary slow response; the secondary response was visible only with stimulus trains of 20 pulses or more. In the presence of LSD we measured the maximum response since we were unable to distinguish the two components. Two points of interest arise; first the response in the presence of LSD was always less than the control twitch amplitude and at low stimulus numbers where the twitch response predominates this appeared as a straight inhibition. With longer trains the response in the presence of LSD was less than the control twitch amplitude but greater than the secondary response which was potentiated (Figure 7). The second point was that, unlike the anococcygeus, the inhibition in the vas deferens was less clearly related to the LSD concentration and had practically reached its maximum at 10^{-8} M.

In the pithed rat the maximum dose of LSD used, 200 µg/kg, had no effect on the motor response to nerve stimulation of the anococcygeus but did inhibit the first 'twitch' phase of the response in the vas deferens. Guanethidine 10 mg/kg completely abolished the entire motor response in both the anococcygeus and the vas deferens.

Effect of LSD on the response to inhibitory nerve stimulation

As Figure 5 shows, LSD was as effective as guanethidine in raising tone and reversing the response to field stimulation from contraction to inhibition.

If tone was first raised with guanethidine so as to convert the motor response to inhibition then the addition of LSD had no effect on these inhibitory responses as Figure 8 illustrates.

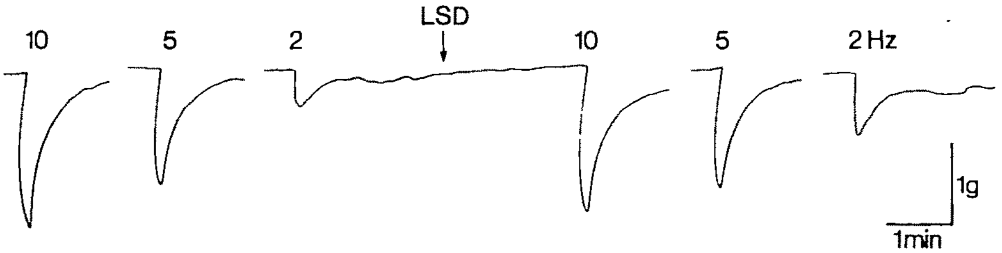

Figure 8 The absence of any effect of lysergic acid diethylamide (LSD, 3 x 10^{-7} M) on the inhibitory response to field stimulation of the rat anococcygeus muscle. In this experiment tone had been raised and the motor nerves blocked by the previous addition of guanethidine 3 × 10^{-5} M. The inhibitory response at three frequencies of stimulation shown above the records are unaltered by the addition of LSD.

Discussion

These results, particularly in the anococcygeus, strongly suggest that LSD can be regarded as an adrenergic neurone blocking agent with an indirect sympathomimetic action. The simultaneous comparison in Figure 5 of the action of guanethidine and LSD on a pair of muscles from the same animal shows identical effects with a rather similar time course. Even the smallness of the contractions produced in the vas deferens accords with an indirect sympathomimetic action since tyramine and guanethidine produce identically small responses in this tissue. That the contractions produced by LSD are due to the release of NA from adrenergic nerves is shown by their sensitivity to phentolamine and to 6-OHDA. That the reduction in the response to motor nerve stimulation is due to adrenergic neurone blockade is suggested by the undiminished response to NA and the lack of effect on the response to inhibitory nerve stimulation; it is also in agreement with the observation of Hughes (1973) that LSD reduces the nerve-stimulated release of NA from the adrenergic nerves of the guinea-pig vas deferens. 6-OHDA was employed to differentiate between the direct and indirect sympathomimetic action of LSD in preference to reserpine pre-treatment since a previous study (Gillespie & McGrath, 1974c) demonstrated that motor nerve responses could still be elicited from both anococcygeus and vas deferens even after the NA content had been reduced to very low levels. We suggest that the NA depletion of the adrenergic nerves by reserpine is incomplete and sufficient NA remains in these reserpine-treated animals both to maintain effective neurotransmission and an indirect sympathomimetic action. Our interpretation relies heavily on the ability of 6-OHDA *in vitro* to produce an effective sympathetic denervation. The technique has been used successfully in several other laboratories (Sachs, 1971; Furness, 1971; Wadsworth, 1973) and in the present investigation we have shown that it abolishes the response to motor nerve stimulation and to indirect sympathomimetic drugs while potentiating the response to NA. We have also found that the fluorescent adrenergic nerve terminals disappear and that they cannot be made to reappear by incubation with NA plus a monoamine oxidase inhibitor, as is possible after reserpine. The ability of LSD to exercise an indirect sympathomimetic action does not, of course, exclude the possibility that it can also in some tissues directly stimulate smooth muscle cells. An example of this is found in the experiments of Dyer & Gant (1973) who have shown that LSD can produce contraction of non-innervated umbilical blood vessels, an effect blocked by bromo-lysergic acid and therefore presumably mediated by 5-HT receptors on the smooth muscle. However, in the vas deferens and the anococcygeus, the complete absence of a motor effect after 6-OHDA and the complete block of the motor effect in normal tissue by phentolamine suggest that direct stimultion of the smooth muscle is negligible.

The ability of methysergide to reduce the motor effect of LSD and 5-HT without influencing the response to NA may indicate that the mechanism of LSD binding to the nerve membrane prior to uptake involves a 5-HT receptor. This might also explain why methysergide is ineffective if added after the response to LSD has fully developed. If the LSD is now inside the nerve varicosity then interference with uptake will not prevent its sympathomimetic action. On this interpretation phentolamine blocks the action of LSD post-synaptically at the α-adrenoceptors while methysergide acts pre-synaptically to block receptors involved in the uptake of LSD.

The hypothesis we put forward will explain all of the actions of LSD on the rat and cat anococcygeus and also the ability to cause contraction of the vas deferens. It does not explain the obvious difference in the vas deferens between guanethidine which abolishes the entire motor nerve response and LSD which inhibits only the initial twitch component. The origin of the two components of this response in the vas is obscure, drugs such as reserpine and phentolamine which have a predictable action on other adrenergically innervated tissue produce a selective block of one or other component of the response in the vas deferens (Swedin, 1971; Gillespie & McGrath, 1974c) and LSD appears to be in a similar category. These exceptions we feel are more a reflection of the unusual nature of the motor response in the vas deferens than a criticism of the mode of action of such drugs on adrenergic neuro-effector transmission generally.

Our thanks are due to the Wellcome Trust for a grant to one of us, to the Medical Research Funds (Rankin Fund) of Glasgow University for a grant for apparatus and to Miss H. McCaffery for skilled technical assistance.

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(Received April 2, 1975.)

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RELEASE OF PROSTAGLANDIN-LIKE SUBSTANCES BY SHIGELLA ENDOTOXIN AND ITS INHIBITION BY NON-STEROIDAL ANTI-INFLAMMATORY COMPOUNDS

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1 The effects of intravenous and intraocular injections of Shigella endotoxin on the inflammatory response, the release of prostaglandin-like substances into the aqueous humour, and the effects of indomethacin on the inflammatory response were studied in the rabbit eye.

2 Both intravenous and intraocular injection of endotoxin released 12.8 ng to 72 ng of prostaglandin-like substances per ml of aqueous humour and increased the permeability of the blood-aqueous barrier as shown by the rise in aqueous humour protein (26-52 mg protein/ml of aqueous humour).

3 Indomethacin, 0.25% administered topically completely inhibited the release of prostaglandin-like substances but was found to have inconsistent inhibitory effects on the clinical signs of inflammation and on the blood-aqueous barrier. Indomethacin was less effective in eyes receiving the higher dose of endotoxin.

4 It is suggested that prostaglandins and possibly other chemical mediators are involved in endotoxin-induced ocular inflammation.

Introduction

Systemic injection of bacterial endotoxin produces a number of haemodynamic changes such as vasodilatation, hypotension and increased vascular permeability (Wiel, McLean, Visscher & Spink, 1956; Eckman, King & Brunson, 1958; Gilbert, 1960). The ocular effects of endotoxins injected either systemically or intraocularly have been studied by a number of workers (Howes, Aronson & McKay, 1970; Gamble, Aronson & Brescia, 1970). Intraocular injection of endotoxin results in a transient rise in intraocular pressure, vasodilatation, breakdown of blood-aqueous barrier and cellular infiltration. In a recent study, Bito (1974) reported migration of white blood cells, severe iritis, decreased ascorbate concentrations and increased protein concentrations (32 mg/ml) in the aqueous humour, 24 h after intravitreal injection of 10 μg of Shigella endotoxin. Chemical substances suggested as mediators of endotoxin reactions are histamine (Kellaway, Trethewie & Turner, 1940; Weil & Spink, 1957), 5-hydroxytryptamine (Armin & Grant, 1957; Davies, McQuarrie & Meeker, 1959), noradrenaline and adrenaline (Thomas, 1954; Howes & McKay, 1972).

In recent years, prostaglandins have been detected in inflammatory exudates from various tissues (Willis, 1969; Giroud & Willoughby, 1970; Greaves, Sondergaard & McDonald-Gibson, 1971; Higgs, Vane, Hart & Wojtulewski, 1974). Eakins, Whitelocke, Perkins, Bennett & Unger (1972) and Eakins, Whitelocke, Bennett & Martenet (1973) demonstrated the release of prostaglandins into the aqueous humour of rabbits and man with acute ocular inflammation.

Studies on the mechanism of action of nonsteroidal anti-inflammatory drugs showed that compounds such as indomethacin and aspirin inhibited prostaglandin synthesis, implying that prostaglandins are involved in the inflammatory process (Ferreira, Moncada & Vane, 1971; Smith & Willis, 1971; Vane, 1971).

The present study examines the release of prostaglandin-like substances by Shigella endotoxin administered intravenously and intraocularly, and the effect of indomethacin on prostaglandin release.

Methods

Adult albino rabbits, weighing 2.5-3.5 kg were used. Shigella endotoxin (Difco) and indomethacin were administered either intravenously or intraocularly.

Intravenous injection

Shigella endotoxin, 100 μg/kg, dissolved in 0.9% w/v NaCl solution (saline) was injected slowly over a period of 3-5 min into the marginal ear vein. Changes in pupil size and blood-vessels were observed for 3-4 h after which time the animals were killed and the aqueous humour removed for assay of prostaglandin-like substances and protein. In separate groups of rabbits, indomethacin, 10 mg/kg (solutions prepared by adding equimolar concentrations of Na_2CO_3 to aqueous suspensions of indomethacin) was injected intravenously 10 min before the endotoxin injection, and aqueous humour was removed as previously. White blood cells in diluted aqueous humour were counted in a haemacytometer.

Intraocular injection

Shigella endotoxin, 2 or 10 μg, was injected in 10 or 50 μl of saline into the anterior chamber or vitreous body respectively, using a No. 30 needle with an Agla micrometer syringe.

Treatment with 100 μl of 0.25% indomethacin, prepared as above, and administered topically, began 2 h before or 12 h after the endotoxin injection, at two hourly intervals for the first 24 h and then, three times a day.

Eyes from a separate group of rabbits, injected intravitreally with 50 μl of saline, were examined, and the aqueous humour was analysed for prostaglandin-like substances and for protein as described below.

Eyes were examined with a slit lamp and vasodilatation aqueous flare, conjunctival oedema and miosis were assessed and scored from 0-4 (0 being normal, 4 being the most severe change). These scores were summed up for each eye and the mean was taken as an 'Inflammatory index'. Photographs of control and treated eyes were taken on Tri-X film using a Nikon F Camera with an 80 mm lens, f 4.5 and a Xenon-flash tube energised by 250 J from a Bowen's Multilec 500.

Twenty-four hours after injection of endotoxin, rabbits were anaesthetized with sodium pentobarbitone and aqueous humour was withdrawn. Protein in the aqueous humour was determined according to the method of Lowry, Rosenbrough, Farr & Randall (1951). Prostaglandin-like substances in the aqueous humour were extracted according to the method of Unger, Stamford & Bennett (1972), assayed against PGE_1 using rat fundus strips suspended in 5 ml of Krebs solution at 37° C, gassed with 5% CO_2 in O_2, and containing methysergide, atropine and mepyramine (all 0.1 μg/ml) and indomethacin (1 μg/ml).

Results

Intravitreal injection of saline into the control eyes did not produce inflammation and did not release prostaglandin-like substances into the aqueous humour. The protein concentrations were in the normal range (0.6-1 mg/ml).

Within 30 min of an intravenous injection of endotoxin, the rabbits became very weak, did not move, and showed signs of endotoxin shock. Aqueous flare, due to exudation of protein into the anterior chamber, vasodilatation and miosis appeared simultaneously. These reactions, expressed as an 'Inflammatory index', reached their peak at about 2 h after the intravenous injection and were less intense than those observed after intraocular injections. Aqueous humour, withdrawn 3 h after the endotoxin injection contained a mean of 12.8 ng/ml prostaglandin-like substances and 26 mg/ml of protein (Table 1).

Table 1 Release of prostaglandin-like substances and increase of protein in aqueous humour following the administration of Shigella endotoxin by various routes.

Route of administration	*Doses of endotoxin*	*Prostaglandin-like substances* ng/ml of aqueous humour (mean ± s.d.)*	*Protein* mg/ml of aqueous humour (mean ± s.d.)*	*Inflammatory index (mean ± s.d.)*
Intravitreal	10 μg	72.6 ± 15 (5)***	50.0 ± 12 (5)	9.8 ± 2.0(20)
	2 μg	47.0 ± 6.5(3)	42.0 ± 8.5(3)	7.0 ± 1.5(12)
Intracameral	10 μg	52.0 ± 14 (3)	32.0 ± 9.9(3)	8.3 ± 1.5(15)
Intravenous**	100 μg/kg	12.8 ± 5.8(4)	26.0 ± 5.5(4)	4.0 ± 1.0(12)

* Each experiment represents pooled aqueous humour from 4-5 rabbits.
** Aqueous humour was withdrawn 3 h after endotoxin.
*** Figure in brackets denotes number of experiments.
s.d. = Standard Deviation.

Intraocular endotoxin

Intracameral .The inflammatory responses of the eye to 10 μg of endotoxin began to appear between 4 and 6 h after injection. These responses, characterised by vasodilatation, miosis and aqueous flare, were most severe after 16 hours. Slit lamp examination showed oedema of the iris, cells and fibrin in the aqueous humour. The mean concentration at 24 h of prostaglandin-like substances in the aqueous humour was 52 ng/ml and that of protein was 32 mg/ml.

Intravitreal. After intravitreal injection of 10 μg of endotoxin, ocular responses were qualitatively similar to those observed after intracameral injection, but the onset of reactions was between 8 and 12 h and the intensity of the responses was greater. In addition to miosis, vasodilatation and cellular infiltration, conjuntival oedema was also evident. The responses peaked after 20 h (Plate 1) and remained so for 48 to 72 h, after which time reactions began to subside very slowly and the eyes returned to apparent normality over 10-15 days. At 24 h the mean concentration of prostaglandin-like substances in the aqueous humour was 72.6 ng/ml. After 2 μg of endotoxin, inflammatory reactions were similar (Plate 2) but milder, and the return to normality was more rapid – over 3-5 days. The mean concentration of prostaglandin-like substances was significantly less than that after 10 μg of endotoxin. After either 2 or 10 μg of endotoxin, protein levels in the aqueous humour were very high, and white cells appeared in the aqueous humour 8 h after the injection.

Inhibitory effects of indomethacin on the responses to:

Intravenous endotoxin. Pretreatment with indomethacin 10 mg/kg (i.v.) 10 min before endotoxin gave almost complete protection against endotoxin shock. Although vasodilatation and miosis were prevented for the first 3 h, mild vasodilatation appeared later. The aqueous humour of these rabbits showed no prostaglandin-like activity, but contained some protein indicating that leakage through the blood-aqueous barrier had not been fully inhibited (Figure 1).

Intraocularly administered endotoxin. When the treatment commenced 2 h before the injection of endotoxin, the effect of indomethacin on inflammatory responses was greater than that of indomethacin treatment begun 12 h after the injection. The inhibition of the 'Inflammatory index' shown in Figure 1 represents the combined results with both types of indomethacin treatment. The release of prostaglandin-like substances into the aqueous humour was completely inhibited by indomethacin. However, indomethacin inhibited the inflammatory response to 10 μg of endotoxin by only 39%, and to 2 μg of endotoxin by 70%; the rise in protein concentration was reduced by 45% and 57% respectively (Figure 1).

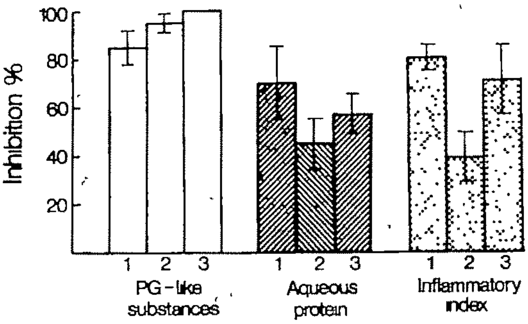

Figure 1 Percentage inhibition of inflammatory responses to endotoxin by indomethacin. Columns 1, 2, 3 represent inhibition of responses to 100 μg/kg (intravenously), 10 and 2 μg (intraocularly) of endotoxin, respectively. Note that although the release of prostaglandin-like substances, 10 and 2 μg of endotoxin was almost completely inhibited, the rise in aqueous humour protein was inhibited by 45-57%, and inflammatory indices by 39-70%.

Comparison of Plates 1 and 3, and 2 and 4 shows that indomethacin suppressed the inflammation produced by 2 μg of endotoxin more effectively than that produced by 10 μg. White cell counts in aqueous humour (predominantly polymorphonuclear cells) were 6000-10,000/mm^3 and 3000-6000/mm^3 after 10 and 2 μg of endotoxin, respectively. Indomethacin had no effect on the migration of white cells into the aqueous humour after 10 μg of endotoxin, and inhibited by 10-15% the migration of white cells into the anterior chamber after 2 μg of endotoxin.

Discussion

Endotoxins do not produce inflammatory reactions by a direct action on target tissues, but through the release of chemical mediators (Gilbert, 1960) such as histamine, 5-hydroxytryptamine polypeptides and prostaglandins.

Histamine is released during the early phase of endotoxin, and other inflammatory reactions (Kun, 1947; Weil & Spink, 1957; Hinshaw, Jordan & Vick, 1961; Spector & Willoughby, 1963, 1968). Antihistamines inhibited changes in vascular permeability only in the initial stages of

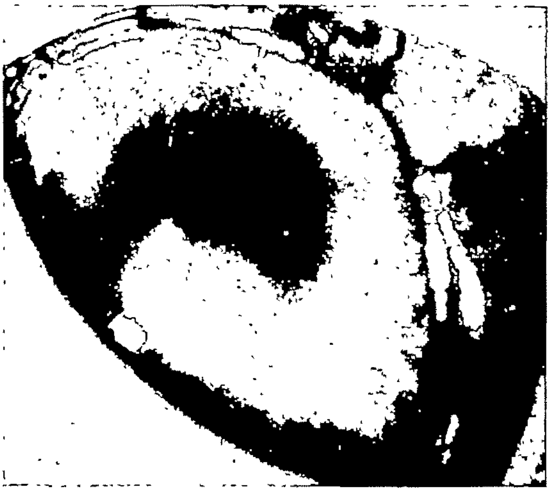

Plate 1 Rabbit eye, 24 h after 10 μg of intravitreal injection of Shigella endotoxin. Note: the detail of the iris cannot be seen due to iris oedema and inflammatory exudate in the anterior chamber.

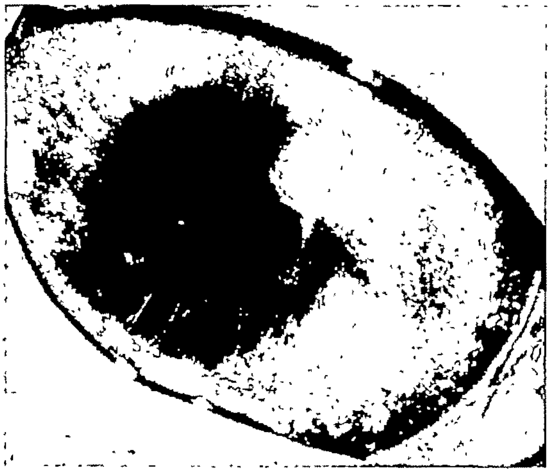

Plate 3 Eye injected as in Plate 1, but pretreated topically with 0.25% indomethacin. Although indomethacin has reduced inflammation, exudate and iris oedema are still present.

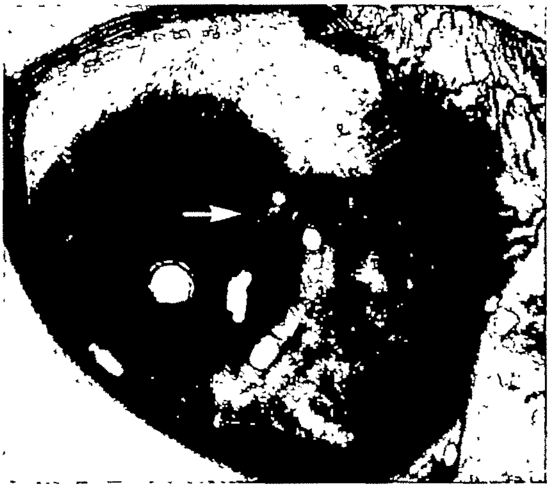

Plate 2 Rabbit eye 24 h after 2 μg of intravitreal endotoxin. Inflammatory exudate in the anterior chamber can be seen (at arrow).

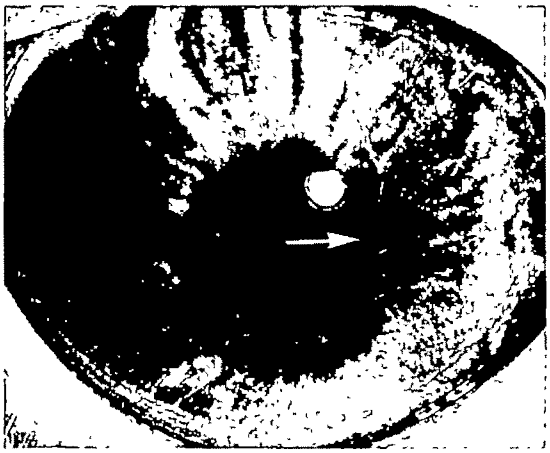

Plate 4 Eye injected as in Plate 2, but pretreated topically with 0.25% indomethacin. Note: almost normal iris and clear anterior chamber except that fibrin (at arrow) still present in the anterior chamber.

inflammation, and despite their repeated administration, increased vascular permeability at later stages could not be suppressed (Spector & Willoughby, 1957, 1959; Wilhelm & Mason, 1960). On the other hand, Schayer (1963) suggested, from studies on histidine decarboxylase activity, that stored histamine was not important, but that its continuous synthesis was responsible for changes in the later phase of inflammation.

Numerous mast cells are normally present in the posterior uvea, but not in the iris (Smelser & Silver, 1963). They migrate to the anterior uvea at an early stage of the inflammation induced by Shigella endotoxin and bovine serum albumin, and degranulate in the later phase of inflammation (Smelser, 1964; Segawa & Smelser, 1969). Histamine has been reported to be released in the initial stage of ocular inflammation (Kapuściński, Baran & Sikorska, 1965), but its role is not yet known.

Activation of a kallikrein-kinin system (Nies, Forsyth, Williams & Melmon, 1968; Erdos & Miwa, 1968) and increased levels of circulating catecholamines after administration of endotoxin, have been reported (Egdahl, 1959). Kinins were found to play only a transitory role in the acute inflammatory process (Di Rosa, Giroud & Willoughby, 1971). Adrenaline is released from the adrenal medulla in the early stages of the reaction to endotoxin. Hinshaw, Brake, Emerson, Jordan & Masucci (1964) reported that adrenalectomized dogs were more sensitive to endotoxin, which suggested that catecholamines play a protective role. Adrenaline and noradrenaline have been reported to suppress inflammation (Setnikar, Subratorra & Temilcou, 1959; Northover & Subramanian, 1961). These studies on the release of chemical mediators by endotoxin were carried out after its intravenous administration.

Intraocular injections of endotoxin produce local inflammatory responses, but the role played by locally available histamine, kinins and noradrenaline is not known. Ocular responses to endotoxin are partially suppressed by superior cervical ganglionectomy and it has been suggested that a high local concentration of catecholamines in the uvea is partly responsible for making the eye unusually sensitive to the effects of endotoxin (Howes & McKay, 1972).

The release of prostaglandins into the aqueous humour of rabbits during acute immunogenic inflammation induced with bovine serum albumin has been reported by Eakins *et al.* (1972). The present study shows that non-immunogenic inflammation produced by endotoxin prostaglandin-like substances are also released into the aqueous humour. However, another mediator (or mediators) seems to be released as well since indomethacin, while inhibiting prostaglandin-release completely, only partially suppressed the clinical signs of inflammation and the increase in vascular permeability.

Indomethacin did not have the same effect on all parameters of the endotoxin response. The systemic hypotension, decreased central venous pressure and cardiac output and metabolic acidosis (Gilbert, 1960; Parratt & Sturgess, 1974) were inhibited by indomethacin as indicated by the good condition of the rabbit after intravenous endotoxin. The suppression of the clinical signs of ocular inflammation was incomplete and seemed to depend on the inhibition of vascular leakage. The fact that inflammatory responses to 2 μg of endotoxin were not much less than those to 10 μg indicated that the latter dose was a very strong stimulus which produced responses beyond effective control by non-steroidal anti-inflammatory compounds.

This work was supported in part by a grant from the Medical Research Council. The author wishes to thank Professor E.S. Perkins for valuable and helpful suggestions; Mr C.F. Higgins and Mr A. Phylactos for excellent technical assistance; Dr John E. Pike, Upjohn Co. Kalamazoo, Michigan, U.S.A. and Messrs Merck Sharp and Dohme Ltd. Hertfordshire, England, for their gifts of prostaglandins and indomethacin.

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(Received February 7, 1975.)

Br. J. Pharmac. (1975), 54, 495-491

REDUCTION OF ANTIGEN-INDUCED CONTRACTION OF SENSITIZED HUMAN BRONCHUS *in vitro* BY INDOMETHACIN

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1 Contraction of passively sensitized human bronchus induced by antigen challenge *in vitro*, was reduced by the prostaglandin $F_{2\alpha}$ synthetase inhibitor, indomethacin.

2 Prostaglandin $F_{2\alpha}$ was released during challenge and this release was inhibited by indomethacin.

3 There was a significant correlation between prostaglandin $F_{2\alpha}$ release and antigen challenge-induced contraction, suggesting that this substance may contribute to the bronchoconstrictor response of sensitized human bronchus to antigen.

Introduction

Ever since the discovery of prostaglandins in human lung (Ånggård, 1965; Karim, Sandler & Williams, 1967) and of the potent bronchoconstrictor effects of prostaglandin $F_{2\alpha}$ in man (Mathé, Hedquist, Holmgren & Svanborg, 1973) there has been speculation about a possible role for prostaglandins in the pathogenesis of asthma. The release of prostaglandins by antigen-challenged human lung *in vitro* (Piper & Walker, 1973) and the presence of a prostaglandin $F_{2\alpha}$ metabolite in the blood of asthmatic patients on inhalation of antigen (Gréen, Hedquist & Svanborg, 1974) gave support to the hypothesis that prostaglandin $F_{2\alpha}$ is a mediator of bronchoconstriction in anaphylaxis. The effect of indomethacin, an inhibitor of prostaglandin $F_{2\alpha}$ synthetase (Vane, 1971), was investigated to establish whether this prostaglandin participates in the contraction of sensitized human bronchus when exposed to antigen *in vitro*.

Methods

Human bronchus 2-3 mm in diameter was dissected free of macroscopically normal lung tissue removed at thoracotomy for bronchial carcinoma, and passively sensitized by overnight storage at 4° C in undiluted pooled serum obtained from patients with asthma due to house dust mite allergy. Sensitized bronchi were cut into spiral strips and one pair from any one lobectomy specimen was suspended in 10 ml organ baths, under a load of 250 mg, perfused with Krebs solution at 37° C and gassed with 95% O_2 and 5% CO_2. Changes in muscle length were measured with an isotonic transducer (SRI) and recorded on moving paper (Rikadenki-Kyogo). Change in the responsiveness of the tissues were detected by parallel control experiments. After about one hour, when the muscle tone was steady maximum responses of test and control preparations to acetylcholine (180-360 μg/ml for 3 min) were obtained and the responses to a standard dose of arachidonic acid (2-10 μg/ml for 5 min) were measured. Indomethacin (3.6 μg/ml) was added to the organ bath and when a steady state of tone had been regained, the test doses of acetylcholine and arachidonic acid were repeated. Finally, each preparation and its paired control was challenged with diluted freeze dried house dust mite antigen (*D. pteronyssius*, 10 μg/ml). Responses to the drugs were expressed as millimetres of pen deflection. The antigen challenge responses were expressed as a percentage of the maximum acetylcholine response (MAR) recorded immediately prior to challenge; in this way results from different tissues could be compared.

The remaining bronchial muscle spirals were used for radioimmunoassay of the prostaglandin $F_{2\alpha}$ released. Parallel controls were carried out, half the preparations being incubated in Krebs solution alone and the remainder in the presence of indomethacin (3.6 μg/ml) throughout the experiment. The spirals were placed in tubes containing 5 ml of gassed Krebs solution at 37° C and were washed three times. They were then incubated for 12 min and the bathing fluid from the tubes was acidified to pH 1-2. extracted with ether and the prostaglandin $F_{2\alpha}$ content measured using the radioimmunoassay method described by Hennam, Johnson, Newton & Collins (1974) giving the basal level of prostaglandin $F_{2\alpha}$. Finally, the bronchial muscle spirals were placed in tubes containing house dust mite antigen (10 μg/ml) and following challenge the prostaglandin $F_{2\alpha}$ was measured as before. Each preparation was dried and weighed and the results expressed as picograms (pg) prostaglandin $F_{2\alpha}$ per mg of bronchial tissue.

In a similar series of experiments the amounts of prostaglandin $F_{2\alpha}$ released during a maximal contraction induced by acetylcholine (360 μg/ml) was measured and this was compared with the amount released by the same tissues on challenge.

Results

All sensitized bronchial spiral preparations contracted on exposure to acetylcholine and arachidonic acid. Indomethacin caused a slow relaxation of the preparations but on attaining a new steady state of tone the response to acetylcholine was unaffected ($P > 0.05$, Student's *t*-test) while the effect of arachidonic acid was reduced ($P < 0.001$) from 22 ± 6 mm (mean ± s.e. mean) to 3 ± 1 mm. The responses to both acetylcholine and arachidonic acid were unchanged in the parallel untreated controls ($P > 0.05$). Antigen challenge resulted in contraction of the bronchial spirals, but the mean response of the indomethacin-treated tissues (74.2 ± 10.3% MAR) was significantly ($P < 0.01$) reduced compared with the untreated controls (119.5 ± 11% MAR).

Radioimmunoassay of prostaglandin $F_{2\alpha}$ revealed slightly higher basal synthesis in indomethacin-treated spirals than in untreated controls ($P > 0.05$). On antigen challenge the mean concentration of prostaglandin $F_{2\alpha}$ in the bathing fluid (± s.e. mean) expressed as pg/mg dry weight of bronchial tissue rose from 25.4 ± 7.3 to 148.1 ± 57.7 (15 experiments, $P < 0.05$). In the indomethacin-treated tissues the amount of prostaglandin $F_{2\alpha}$ decreased on antigen challenge from 51.1 ± 14.6 to 47.9 ± 10.6 pg/mg ($P > 0.05$). There was a significant correlation between percentage change in organ bath prostaglandin $F_{2\alpha}$ concentrations on antigen challenge and contraction of the paired tissues in the organ bath ($r = 0.61$, $P < 0.05$, method of least squares).

A dose of acetylcholine which induced a maximal contraction of the bronchial spirals in the organ bath caused a slight but not significant increase in the amount of prostaglandin $F_{2\alpha}$ released, from 27.0 ± 8.2 to 3.7 ± 8.1 pg/mg ($P > 0.05$). In the same preparations antigen challenge caused a mean increase from 29.4 ± 6.1 to 117.4 ± 16 pg/mg prostaglandin $F_{2\alpha}$ ($P < 0.001$).

Discussion

The results of the experiments reported in this paper provide further evidence in favour of the hypothesis that prostaglandin $F_{2\alpha}$ synthesis contributes to anaphylactic bronchoconstriction. Organ bath concentrations of indomethacin, sufficient to inhibit contractions elicited by the prostaglandin precursor arachidonic acid, reduced the antigen-induced contraction of human bronchi by 40% compared with paired controls. Parallel experiments indicated that the synthesis of prostaglandin $F_{2\alpha}$ increased during antigen challenge and that there was a significant correlation between response to antigen challenge and percentage change in prostaglandin $F_{2\alpha}$ organ bath concentrations. Complete inhibition of prostaglandin $F_{2\alpha}$ synthesis by indomethacin did not completely abolish antigen-induced bronchial muscle contraction, indicating that it only contributed to the antigen response.

Release of prostaglandin $F_{2\alpha}$ during bronchospasm could be a secondary event following muscle contraction such as occurs in the guinea-pig trachea (Orehek, Douglas, Lewis & Bouhuys, 1973) but since there was no significant increase in the amount of prostaglandin $F_{2\alpha}$ synthesized by human bronchus during contractions induced by acetylcholine of the same order as those induced by antigen this explanation seems unlikely. Alternatively, prostaglandin $F_{2\alpha}$ released during anaphylaxis could act by sensitizing bronchial muscle to the other mediators released such as histamine and slow reacting substance (SRS)-A, but definite exclusion of these possibilities awaits results of experiments in progress. Although its mode of action remains in doubt, it appears that prostaglandin $F_{2\alpha}$ synthesis does contribute to the antigen-induced contractile response of sensitized human bronchus *in vitro*.

This investigation was financed by a Medical Research Council Programme Grant. We are indebted to the thoracic surgeons who kindly supplied us with human bronchus; to Mr W.A. Slater of Merck Sharp and Dohme Limited for indomethacin; to Dr P. Roylance of Beechams Laboratories for house dust mite antigen; to Dr J. Pike of Upjohn Limited for supplies of prostaglandins. We thank the Department of Obstetrics and Gynaecology, King's College Hospital for assistance with the radioimmunoassay.

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(Received April 14, 1975.
Revised May 12, 1975.)

Br. J. Pharmac. (1975), 54, 499-505

PROSTAGLANDIN RELEASE FROM CAT AND DOG SPLEEN

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1 The output of prostaglandins from the spleens of cats and dogs was studied. Comparison is made between the results found in the two species.

2 The release of prostaglandins was investigated in isolated saline-perfused spleens and in incubates of spleen slices. Release in response to nerve stimulation, and exposure to adrenaline or noradrenaline was compared with resting release.

3 A resting release of prostaglandins was found in the dog but not in the cat spleen.

4 Whereas stimulated dog spleens released microgram quantities of prostaglandins E_2 and $F_{2\alpha}$, prostaglandin output from the cat spleen under similar conditions was undetectable or barely detectable.

5 The identity of the prostaglandins released from the dog spleen (prostaglandins E_2 and $F_{2\alpha}$) was confirmed by mass spectrometry.

6 The species difference in prostaglandin output from the spleen is discussed in relation to the hypothesis that endogenous prostaglandins modify the responses of this organ to nervous stimuli.

Introduction

The dog spleen was among the first of several organs and tissues shown to release prostaglandin-like material in response to autonomic nerve stimulation (Davies, Horton & Withrington, 1967; Ferreira & Vane, 1967). Others include heart (Wennmalm & Stjärne, 1971; Samuelsson & Wennmalm, 1971), kidney (Dunham & Zimmerman, 1970; Davis & Horton, 1972; McGiff, Crowshaw, Terragno, Malik & Lonigro, 1972), adipose tissue (Shaw & Ramwell, 1968), stomach (Bennett, Friedmann & Vane, 1967; Coceani, Pace-Asciak, Volta & Wolfe, 1967), and vas deferens (Hedqvist & von Euler, 1972). Since exogenous prostaglandins modify the responses of various organs to adrenergic nerve stimulation (see review by Brody & Kadowitz, 1974), it is possible that endogenous prostaglandins play a physiological role in the responses of such organs to autonomic control. In testing this hypothesis in any one organ, it is useful to establish whether it releases prostaglandins on stimulation of its autonomic nerve supply.

The cat spleen has been classed with the dog spleen as releasing 'large amounts of prostaglandin' (Hedqvist, 1970a) but there is evidence that the spleens of these two species are not comparable in their ability to release prostaglandins in response to nerve stimulation (Ferreira, Moncada & Vane, 1973; Peskar & Hertting, 1973; Hoszowska & Panczenko, 1974; cf. Davies, Horton & Withrington, 1968; Gilmore, Vane & Wyllie, 1968; Ferreira, Moncada & Vane, 1971). In this investigation, we have measured the quantities of prostaglandins released from cat and dog spleens, at rest and during sympathetic nerve stimulation or exposure to adrenaline or noradrenaline. The stimulated dog spleen released microgram quantities of both prostaglandin E_2 and $F_{2\alpha}$, whereas prostaglandin release from the cat spleen, measured under comparable conditions, was barely detectable.

Methods

Saline-perfused cat and dog spleens

Eight cats and four dogs of either sex were anaesthetized with pentobarbitone sodium, 40 mg/kg i.v. (dogs) or i.p. (cats). The abdomen was opened, and the spleen was freed of all vascular connections except for the splenic artery and vein in the splenic pedicle. These were carefully dissected free from surrounding connective tissue. Each animal was then given heparin (1000 i.u./kg i.v.) and polyethylene cannulae were inserted in the splenic artery and vein. Perfusion was started at a rate of 10-15 ml/min (cat) or 15-30 ml/min (dog), with Krebs-Henseleit solution containing 0.4 to 2% dextran (MW 70,000), maintained at 37° C and aerated with 5% CO_2 in O_2.

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The spleen was transferred to a perfusion chamber and stimulating electrodes were placed in contact with the splenic nerve plexus surrounding the splenic artery. Changes in spleen volume were detected as changes in weight of the whole perfusion chamber using a Grass force-displacement transducer. Perfusion pressure was monitored by a Statham pressure transducer (P.23 Dc).

Nerve stimulation was carried out at 10-40 V, at frequencies of 10-20 Hz and a pulse duration of 2 ms. Solutions of adrenaline or noradrenaline were infused into the arterial cannula at a rate of 1-2 μg/min, with a Braun infusion pump.

In most experiments, samples of perfusate were collected and centrifuged (500 *g* for 10 min at 4° C) to precipitate red blood cells. The supernatant was extracted for prostaglandins.

Incubated spleen slice experiments

Spleens from one cat and two dogs (killed by an overdose of pentobarbitone) were cut into slices 0.7 mm thick and distributed among incubation flasks containing 5 vol. Krebs-Henseleit solution. Adrenaline or noradrenaline was added to 'test' flasks in a final concentration of 0.2 mM. After incubation at 37° C for 1 h, the tissue was removed by filtration and the supernatant extracted for prostaglandins.

Extraction of prostaglandins

Samples of perfusate and incubation media were titrated to pH 4.5 with 1 N HCl, clarified by centrifugation if necessary, and extracted twice with 2 vol. of ethyl acetate. This extract was evaporated to dryness, and the residue taken up in 30 ml 67% ethanol. The ethanolic solution was washed twice with 15 ml of petroleum spirit (b.p. 40-60° C), then evaporated to dryness. Prostaglandins in this final extract were separated from each other and from accompanying impurities by column or thin layer chromatography.

Separation of prostaglandins by silicic acid column chromatography

The dry residue from the solvent extraction procedure was dissolved in 1 ml of 10% ethyl acetate in benzene and applied to columns of silicic acid (4.5 g Sigma Sil-R activated at 110° C for 1 h) 1 cm in diameter. Columns were eluted at a rate of approximately 1 ml/min with increasing concentrations of ethyl acetate in benzene, namely: 10% (40 ml), 40% (75 ml), 65% (130 ml + 20 ml), 80% (150 ml) and 100% (40 ml). Prostaglandins A (not detected in the present experiments) were eluted by 40% ethyl acetate, E by 65%, and F by 80%.

All eluates from the columns were evaporated to dryness and dissolved in 1 ml distilled water for bioassay.

Separation of prostaglandins and contaminating material by thin layer chromatography

Prostaglandins extracted from the incubation medium in the spleen slice experiments were separated by thin layer chromatography (TLC) on 0.25 mm layers of prepared neutral silica gel, using the 'G.C.M.' solvent system (Millar, 1974). This system separates prostaglandins A, E, and F from each other and from contaminating blood pigment which accompanies the prostaglandins through the extraction procedure. TLC was also used to replace column chromatography in two of the perfused spleen experiments: cat no. 5, and dog no. 2.

Bioassay of prostaglandins

Samples obtained from thin layer or column chromatography were bioassayed on the rat stomach strip. Prostaglandins E_1 and $F_{2\alpha}$ were used as bioassay standards in most experiments (prostaglandin E_1 being more readily available than E_2). However, after mass spectrometry had shown that the dog spleen released prostaglandin E_2 as opposed to E_1, prostaglandin E_2 was used as the assay standard (as indicated in the results from spleen slice experiments).

In some experiments, a portion of the venous effluent was assayed directly by superfusing it over a rat stomach strip. A peristaltic pump was used to deliver the effluent from the spleen (maintained at 37° C) to the tissue at a rate of 10 ml/minute. Calibrating doses of prostaglandin E_1 standards were infused into the effluent as appropriate. In these experiments, a solution of antagonist drugs, which was superfused over the tissue simultaneously, was used to increase the specificity of the assay. This solution contained, per litre of

Krebs solution: 2-bromolysergic acid diethylamide 2 mg, hyoscine hydrobromide 0.88 mg, mepyramine maleate 0.84 mg, phenoxybenzamine hydrochloride 6.7 mg and propranolol hydrochloride 6.8 mg. These concentrations had been found by previous experiment to render the tissue insensitive to 5-hydroxytryptamine, acetylcholine, histamine, adrenaline and noradrenaline, while the tissue's sensitivity to prostaglandins was retained.

Identification of released prostaglandins by combined gas chromatography–mass spectrometry

The techniques for preparing derivatives of prostaglandins and their separation and identification by combined gas chromatography–mass spectrometry have been described by Thompson, Los & Horton (1970). Prostaglandin E's were first converted to the corresponding B's by exposure to 0.1 N KOH in methanol for 1 h at room temperature. The solution was then diluted with water, acidified to pH 5 and extracted twice with ethyl acetate. The extract was evaporated to dryness and transferred (using small volumes of methanol) to Eppendorf tubes prior to methylation and formation of the trimethylsilyl ethers. The prostaglandin $F_{2\alpha}$ derivative used was also the trimethylsilyl ether of the methyl ester. Analyses were performed on an LKB 9000 gas chromatograph–mass spectrometer.

Results

Recoveries

The recoveries of authentic prostaglandins E_1 and $F_{2\alpha}$ after the extraction and column chromatographic procedures used in the majority of the perfusion experiments were 30% and 40% respectively (means of three determinations).

In two of the perfusion experiments, column chromatography was replaced by TLC. In one of these experiments (Table 1, cat no. 5), recoveries of prostaglandins E_1 and $F_{2\alpha}$ were monitored by the addition of trace amounts of tritium labelled compounds to the perfusate. Recoveries of the two prostaglandins were 50% and 53% respectively. In the second such experiment (dog no. 2) recoveries were not determined, but were assumed to be 50% for prostaglandin E and 60% for F, on the basis of recoveries found in other experiments where prostaglandins were separated by TLC.

In the spleen slice experiments, recoveries were determined using tritiated prostaglandins E_1 and F_{2a} which were added to the supernatant from each flask after the incubation. Mean recoveries from the three experiments were 50% for prostaglandin E_1 and 68% for F_{2a}.

All the results which follow have been corrected for recovery.

Prostaglandin release from perfused spleens

Resting output The results are shown in Table 1. Perfusate from the resting spleen was collected immediately before the stimulation period (see below) and for the same duration. No prostaglandin was detected in the effluent from unstimulated cat spleens ($\nless 0.26$ ng g^{-1} min^{-1} of either prostaglandin E_1 or $F_{2\alpha}$). However, a small

Table 1 Prostaglandin release from saline-perfused cat and dog spleens, at rest and in response to splenic nerve stimulation

Species and experiment number	*Weight of spleen (g)*	*Prostaglandin released**						*Nerve† stimulation*
		At rest		*Stimulated*				
		PGE (as E_1)	*PGF (as $F_{2\alpha}$)*	*PGE (as E_1)*		*PGF (as $F_{2\alpha}$)*		
Cat No. 1	30	<0.16	<0.25	<0.14	*<0.46*	<0.13	*<0.42*	(a)
2	16	<0.06	<0.10	<0.06	*<0.22*	<0.10	*<0.32*	(a)
3	24	<0.11	<0.13	0.13	*0.43*	<0.13	*<0.43*	(a)
4	20	<0.07	<0.26	<0.07	*<0.30*	<0.26	*<1.20*	(b)
5	25	<0.24	<0 21	<0.20	*<0.68*	*<0.14*	*<0.48*	(c)
Dog No. 1	56	0.52	0.67	4.02	*13.40*	1.45	*4.83*	(d)
2	64	0.39	0.31	6.25	*20.80*	1.56	*5.20*	(e)

* Figures in italic type are output values expressed as nanograms of prostaglandin released per kilogram of spleen per nerve stimulus (ng kg^{-1} $stimulus^{-1}$). All other output values (figures not in italic type) are expressed as nanograms of prostaglandin released per gram of spleen per minute of collection period (ng g^{-1} min^{-1}).

† Nerve stimulation was carried out as follows: (a) 12,000 stimuli in 2 trains of 10 min each, (b) 8700 stimuli, in 8 trains of 1-2 min each, (c) 3000 stimuli in 1 train of 5 min, (d) 12,000 stimuli in 8 trains of 2-3 min each, (e) 6000 stimuli in 2 trains of 5 min each.

resting output (0.31-0.67 ng g^{-1} min^{-1}) of both prostaglandin E and F was detected in effluent from dog spleens.

Stimulated output

(a) *Nerve stimulation* Stimulation of the splenic nerves (carried out between 1 and 3 h after the start of perfusion) produced a rise in perfusion pressure and a contraction of the splenic capsule. The total 'stimulated' collection consisted of effluent collected during the period of stimulation and during an equal time thereafter. The results are shown in Table 1, where prostaglandin output is expressed both as ng g^{-1} min^{-1} (for comparison with control values in the same experiment) and as ng kg^{-1} stimulus^{-1} (for comparison between experiments and species). Various patterns of nerve stimulation were tried as indicated

No prostaglandin F was detected in effluent from the five stimulated cat spleens. In only one cat experiment (cat no. 3) was a release of prostaglandin E detectable. In marked contrast, the stimulated dog spleens released microgram quantities of both prostaglandins E and F.

(b) *Noradrenaline stimulation* The effect of noradrenaline was investigated in two cat spleens and one dog spleen. Perfusate was collected for 40-55 min during which time the spleens were stimulated repeatedly by noradrenaline infusions (1-2 ng/minute). The noradrenaline evoked large contractile and pressor responses. No detectable prostaglandin was recovered from the perfusate in the first cat experiment. The second cat spleen released 0.13 ng g^{-1} min^{-1} prostaglandin E (as E$_1$) and 0.21 ng g^{-1} min^{-1} prostaglandin F (as F$_{2\alpha}$). A dog spleen similarly treated released 1.2 ng g^{-1} min^{-1} prostaglandin E (as E$_1$) and 0.65 ng g^{-1} min^{-1} prostaglandin F (as F$_{2\alpha}$).

(c) *Examination of effluent by superfusion over rat stomach strips* This was carried out in experiments with one dog spleen and three cat spleens. The results are shown in Figure 1. The dog spleen released prostaglandins on stimulation of the splenic nerves, evidently in microgram quantities as judged by the response of the assay tissue. Of the three cat spleens (all of which were perfused for at least 3 h) only one showed signs of prostaglandin release in response to stimulation. This occurred after 2-3.5 h perfusion when both adrenaline and nerve stimulation evoked the release of a substance that contracted the assay tissue. Calibrating infusions of prostaglandin E$_1$ indicated that the concentration of this substance in the effluent was equivalent to approximately 1 ng/ml prostaglandin E$_1$.

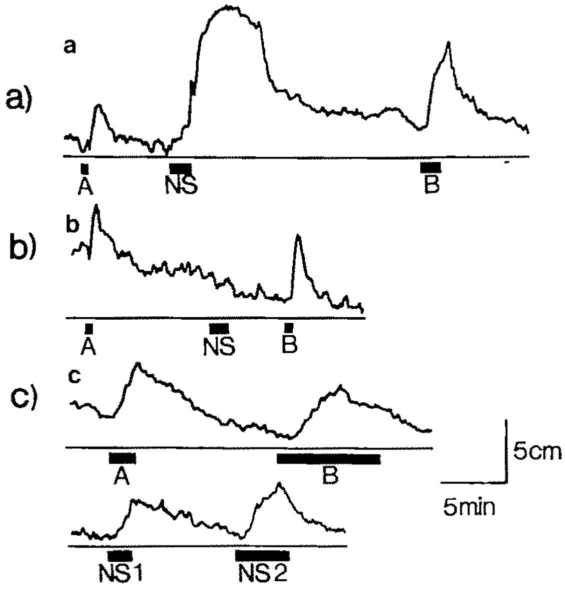

Figure 1 Traces of tension developed in rat stomach strips superfused with effluent from perfused cat and dog spleens. (a) Responses to effluent from a dog spleen, 1 h after the start of perfusion. At A, a calibrating dose of 50 ng prostaglandin E$_1$ was injected over 15 s into the venous effluent. At NS, the splenic nerves were stimulated at 20 Hz (40 V, 2 ms) for 1.5 minutes. A large contracture of the assay strip resulted. This was not matched by 650 ng prostaglandin E$_1$ injected (at B) into the venous effluent over 1.5 min ($\equiv$43 ng/ml). (b) Responses to effluent from a cat spleen, 1 h after the start of perfusion. The assay tissue responded to 20 ng prostaglandin E$_1$ infused over 20 s at (A) and (B), but nerve stimulation (NS, 20 Hz, 40 V, 2 ms) for 1.5 min failed to evoke the release of smooth muscle stimulating material. (c) Responses to effluent from another cat spleen. Upper trace—(2.25 h after the start of perfusion). At A, prostaglandin E$_1$ was infused into the venous effluent for 2 min to give a final concentration of 1 ng/ml. At B, adrenaline was infused into the splenic arterial cannula for 8 min to give a final concentration of 500 ng/ml. This caused the appearance in the venous effluent of a substance that contracted the stomach strip. Lower trace—(3.5 h after the start of perfusion). Nerve stimulation (10 Hz, 40 V, 2 ms) for 2 min (NS1), and 4 min (NS2) caused release of a substance that contracted the stomach strip.

(d) *Identification by GLC-MS of prostaglandins released by the dog spleen* The prostaglandin E- and F-like material isolated chromatographically from the dog spleen perfusate (dog no. 1) collected during the 'stimulated' period was analysed further by gas chromatography–mass spectrometry. Before the preparation of derivatives, the prostaglandin E-like material was converted to prostaglandin B.

Authentic prostaglandin $F_{2\alpha}$ methyl ester/trimethylsilylether (Me/TMS) gave eleven major peaks of m/e values above 300, at 584 (M^+), 569 (M−15), 513 (M−71), 494 (M−90), 423 (M−(90 + 71)), 404 (M−(2 x 90)), 379 (M−(114 + 90 + 1)), 353 (M−141 + 90), 333 (M−(2 x 90 + 71)), 314 (M−(3 x 90)) and 307. The presence of these eleven peaks, in similar ratios in the mass spectrum of the experimental prostaglandin F-like material (Me/TMS) confirmed that this material was prostaglandin $F_{2\alpha}$. There was no indication of the presence of prostaglandin $F_{1\alpha}$.

Authentic prostaglandin B_2 Me/TMS gave 9 prominent peaks of m/e values above 200, at 420 (M^+), 405 (M−15), 389 (M−31), 349 (M−71), 330 (M−90), 321 (M−(71 + 28)), 279 (M−141), 247 (M−173), and 221 (M−199). The presence of these 9 peaks, in similar ratios, in the mass spectrum of the experimental prostaglandin E-like material (after conversion to prostaglandin B Me/TMS) confirmed that this material was prostaglandin E_2. There was no indication of the presence of prostaglandin E_1.

Prostaglandin release from incubated spleen slices

This was investigated in experiments with one cat and two dog spleens. The results are shown in Table 2. Adrenaline had no effect on prostaglandin release from cat spleen slices, but adrenaline and noradrenaline caused a large increase in prostaglandin E output, and a smaller increase in F output from dog spleen slices. Catecholamine-stimulated dog spleen tissue yielded approx. 18 times more prostaglandin E per gram than similarly treated cat spleen tissue. Incubation of dog spleen slices with tritiated prostaglandin E_1 and $F_{2\alpha}$ revealed no detectable metabolism of these compounds under the conditions of the above experiments.

Metabolism of prostaglandin E_1 by the perfused cat spleen

Tritium-labelled prostaglandin E_1 (1 μCi/100 μg) was infused into the inflow cannula of a perfused cat spleen, at a rate of 8 μg/min for 38 minutes. The spleen used was particularly large (110 g) and was thus comparable in mass to a moderately large dog spleen. Chromatographic analysis of the material present in the effluent collected during the infusion and for 1 h thereafter showed that 96.5% of the radioactivity co-chromatographed with standard prostaglandin E_1, and only 3.5% appeared as a less polar metabolite (not identified). Thus there was no evidence for efficient metabolism of this prostaglandin by the cat spleen.

Discussion

We have found that cat and dog spleens, subjected to the same treatment, show marked differences in their ability to release prostaglandins in response to nerve stimulation or catecholamines. This was apparent both in experiments where intact spleens were perfused with a saline solution, and in experiments involving the incubation of spleen slices.

The release of prostaglandins from cat and dog spleens has been investigated by other workers, but no direct comparisons between these species have been made hitherto. Ferreira *et al.* (1973) using the saline-perfused cat spleen detected no basal or stimulated release of prostaglandin during the first hour of perfusion. Thereafter, a basal release of up to 4 ng/ml (prostaglandin E_2 equivalents) was found. During nerve stimulation, prostaglandin release was less than 1 ng/ml in the first few trials, but increased to 10-30 ng/ml after perfusion for 2-3 hours. The authors discuss this increasing release of prostaglandin with time as a possible expression of the gradual deterioration of

Table 2 Prostaglandin release from incubated slices of cat and dog spleen, and the effect of adding adrenaline or noradrenaline

Species and experiment number	*Prostaglandin release (ng g tissue^{-1} h^{-1})*			
	without catecholamine		*with catecholamine*	
	PGE (as E_2)	*PGF (as $F_{2\alpha}$)*	*PGE (as E_2)*	*PGF (as $F_{2\alpha}$)*
			Adrenaline, 0.2 mM	
Cat	15	Traces (<10)	15	Traces (<10)
Dog No. 1	54	27	275	32
			Noradrenaline, 0.2 mM	
Dog No. 2	80	15	280	50

the isolated perfused spleen. We too found, in one experiment, prostaglandin release only after prolonged perfusion of the cat spleen, but we obtained no concentrations as high as those quoted by Ferreira *et al.* Our results agree more closely with those of Peskar & Hertting (1973), who found, using radioimmunoassay, a basal release of prostaglandin E from the cat spleen ranging from undetectable to not more than 0.1 ng/ml, even after 3 h of perfusion. These authors did not investigate the effect of nerve stimulation, adrenaline or noradrenaline on prostaglandin output. However, in a 'representative experiment' angiotensin produced a peak release of only 0.35 ng/ml prostaglandin E and 0.2 ng/ml prostaglandin F during a substantial splenic contraction and rise in perfusion pressure. Hoszowska & Panczenko (1974), in experiments of short duration with saline-perfused cat spleens, found no basal release of prostaglandins, but a release of 1.3 ± 0.1 ng/ml of prostaglandin-like material (measured by superfusion over a rat stomach strip) in response to nerve stimulation.

All the perfused dog spleens examined in the present experiments released microgram quantities of prostaglandins E and F on stimulation, in circumstances where the cat spleen had released undetectable or barely detectable quantities of these compounds. The identity of these prostaglandins, characterized previously only by behaviour on thin layer chromatography and parallel bioassay, has now been confirmed as prostaglandins E_2 and $F_{2\alpha}$ by mass spectrometry. The amounts of prostaglandins we detected in dog spleen perfusates are comparable to those found by other workers using similar techniques (Gilmore, Vane and Wyllie, 1968; Ferreira *et al.*, 1971).

The species difference found in the perfusion experiments was also evident in our incubated spleen slice experiments. Incubation with adrenaline or noradrenaline considerably augmented prostaglandin (particularly prostaglandin E) release from dog spleen slices, but adrenaline had no effect on prostaglandin release from cat spleen slices.

The release of prostaglandins from the spleen may reflect the net result of two processes: synthesis and metabolism. Our experiments do not show definitively which of these processes differs markedly between spleens from cats and dogs. The perfused cat spleen, however, did not appear to be very efficient at metabolizing exogenous prostaglandin E_1. It seems unlikely, therefore, that the large species difference in splenic prostaglandin output is due to a more rapid inactivation of endogenously synthesized prostaglandins in the cat.

The apparent inability of the cat spleen to synthesize prostaglandins in amounts comparable to those produced by the dog spleen might result from a deficiency of prostaglandin synthetase enzymes. Alternatively, it might reflect a paucity of fatty acid prostaglandin precursors, or of the phospholipases necessary for their mobilization prior to prostaglandin synthesis. Experiments with broken cell preparations would help to distinguish between these possibilities.

Cat and dog spleens differ not only in their ability to release endogenous prostaglandin, but also in their susceptibility to exogenous prostaglandins. The responses of the cat spleen to sympathetic nerve stimulation were modified in the presence of exogenous prostaglandins E and prostaglandin synthetase inhibitors (Hedqvist & Brundin, 1969; Hedqvist, 1969, 1970a, 1970b; Hedqvist, Stjärne & Wennmalm, 1971; Ferreira *et al.*, 1973). The responses of the dog spleen, on the other hand, were unaffected by prostaglandins E_1, E_2 or $F_{2\alpha}$ (Davies & Withrington, 1968, 1971). If endogenous prostaglandins play a physiological role in the sympathetic control of spleen function, as proposed by Hedqvist (1970a), it may be that the different degrees of sensitivity of the two species to prostaglandins are correlated with their relative abilities to produce these compounds.

We should like to thank: Professor E.W. Horton for his advice and support; Dr A. Brash for his advice and assistance; Dr C.J. Thompson and Mr C.N. Goode for their assistance with the mass spectrometry; and Dr J.E. Pike of the Upjohn Company, Kalamazoo, Michigan, U.S.A., for supplying the pure prostaglandins. The work was supported in part by a grant to Professor Horton from the Wellcome Trust.

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(Received January 29, 1975.
Revised April 7, 1975.)

Br. J. Pharmac. (1975), 54, 507-509

DISPLACEMENT BY METIAMIDE OF THE DOSE-RESPONSE CURVES TO PENTAGASTRIN AND METHACHOLINE IN THE CONSCIOUS RAT

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1 The effect of a specific histamine H_2-receptor antagonist, metiamide, on the acid dose-response curves for pentagastrin or methacholine was studied in rats provided with Heidenhain pouches.

2 Metiamide induced a shift to the right of the dose-response curve to pentagastrin and lowered the maximal secretion.

3 In contrast, metiamide only increased the ED_{50} for methacholine but did not alter the calculated maximal response.

4 The effect of metiamide on pentagastrin and methacholine-induced secretion is in agreement with the previously suggested difference in the part played by mucosal histamine in the mode of action of the two stimulatory agents.

Introduction

The recently discovered histamine H_2-receptor antagonists (Black, Duncan, Durant, Ganellin & Parsons, 1972; Black, Duncan, Emmett, Ganellin, Hesselbo, Parsons & Wyllie, 1973) have become important in attempts to determine the part played by gastric mucosal histamine in the process of exciting the parietal cell. From experiments mainly in the rat it has been proposed that gastrin evokes acid secretion by means of mobilizing mucosal histamine (see Kahlson, Rosengren & Svensson, 1973). The stable choline esters are considered to activate the parietal cells directly (Rosengren & Svensson, 1969); yet their secretory effectiveness appears dependent on small amounts of histamine continuously reaching the parietal cells (Johansson, Lundell, Rosengren & Svensson, 1972; Lundell, (1975a). In the present study an attempt was made to characterize the inhibition produced by a histamine H_2-receptor antagonist on pentagastrin or methacholine-induced acid secretion in order to obtain further information on the involvement of mucosal histamine in the mode of action of these stimuli.

Methods

Female rats of the Sprague-Dawley strain, weighing about 250 g, were used. They were fed a standard pellet diet and tap water and when provided with pouches had the choice of Tyrode solution or tap water. Heidenhain pouches were prepared in the main according to Alphin & Lin (1959) in fasted rats, anaesthetized with ether. A postoperative period of two months was allowed. Before each experiment, the rats were fasted for 18 h and they were then kept restrained in Bollman cages. Gastric juice was collected in 30 min samples by a perfusion technique (Svensson, 1970) and analysed for HCl by titration against 0.1 N NaOH with phenol red as an indicator. The secretory stimulants and metiamide (N-methyl-N′[2[(5-methyl imidazol-4yl)methyl thio] ethyl] thiourea) were infused via a polyethylene tube inserted in a tail vein, the tube being connected to a motor-driven syringe. In establishing dose-response curves for pentagastrin (Peptavlon ICI 50.123) and methacholine (methacholine chloride, Fluka AG) each dose was infused for 90 min in stepwise increasing doses. The acid output was calculated from the mean of the last two 30 min periods at each dose level. The infusion of metiamide started 1 h before the lowest dose of the stimulatory compound was added. The experiments with pentagastrin or methacholine alone or combined with metiamide were performed in a randomized order.

Results

The effect of metiamide on the dose-response curves for pentagastrin and methacholine was investigated in six rats provided with Heidenhain

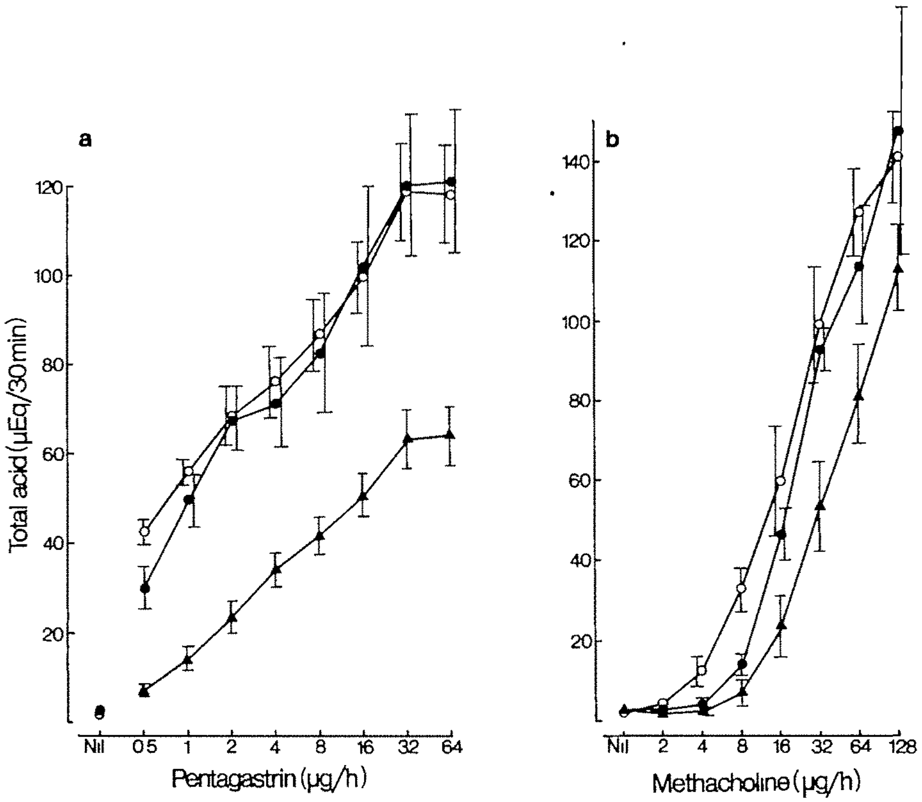

Figure 1 Acid secretion (µEq/30 min) in response to stepwise increasing doses of (a) pentagastrin or (b) methacholine alone (○) or one of the two compounds in combination with 50 µg/h (●) or 500 µg/h of metiamide (▲). Nil indicates interdigestive secretion. The mean and the s.e. mean are calculated from one determination in each of six Heidenhain pouch rats.

pouches. Interdigestive secretion was 2.4 ± 0.32 (s.e. mean) µEq/30 minutes. Infusion of stepwise increasing doses of pentagastrin induced graded secretory responses with an observed maximum of 119.3 ± 10.79 µEq/30 min on infusing 32 µg/h (Figure 1a). By application of Michaelis-Menten equations on the observed responses analogous to the equations used for enzyme reactions (Dowd & Riggs, 1965) the theoretical maximal responses were obtained as well as the ED_{50} values. The calculated maximal response for pentagastrin alone was 118.7 ± 11.38 µEq/30 min which did not significantly differ from the observed response. Metiamide in a dose of 500 µg/h reduced the observed and calculated maximal response to 64.1 ± 6.50 and 66.4 ± 7.40 µEq/30 min respectively.

The effect of metiamide on methacholine-induced acid secretion differed from that on pentagastrin (Figure 1b). Maximal responses could not be obtained in the present study since infusion of higher doses of methacholine than 128 µg/h was followed by adverse side effects. The ED_{50} value and calculated maximal response for methacholine alone were 24.1 ± 9.99 µg/h and 175.8 ± 24.53 µEq/30 min respectively. Neither 50 nor 500 µg/h of metiamide altered the calculated maximal response for methacholine but 50 µg/h metiamide inhibited ($P < 0.05$) the secretory response to the lower doses (2-8 µg/h) of methacholine and 500 µg/h increased the ED_{50} for methacholine from 24.1 ± 9.99 to 90.8 ± 23.3 µg/hour.

Discussion

Metiamide has been characterized as a competitive inhibitor of histamine-induced acid secretion in the rat and dog (Parsons, 1973). In the rat, infusion of increasing doses of pentagastrin mobilizes increasing amounts of histamine from the gastric mucosa until a maximum is reached. The dose of pentagastrin which evokes maximal acid secretion corresponds to that which mobilizes mucosal histamine at a maximal rate (Lundell, 1974; Lundell, 1975b). In the present study, metiamide in a dose of 500 μg/h shifted the dose-response curve for pentagastrin to the right with a reduction of the maximal response. These results are compatible with the hypothesis that pentagastrin is not a direct stimulant of the parietal cell but depends on an intermediary mechanism, namely, the mobilization of histamine.

Metiamide also inhibited the secretion evoked by methacholine but the inhibition differed from that seen with pentagastrin. No difference was noted between the calculated maximal responses obtained with methacholine alone and in combination with the two doses of metiamide, but a progressive increase in the ED_{50} for methacholine was recorded. These results should not be taken as evidence that methacholine activates the H_2-receptor on the parietal cell but rather to show that metiamide blocks the action of histamine mobilized during the interdigestive state, thus interrupting the facilitating relationship that already exists between small amounts of histamine and methacholine (Johansson *et al.*, 1972). The failure of metiamide to reduce the calculated maximal response to methacholine may be accounted for by an increased release of antral gastrin and a subsequent mobilization of mucosal histamine. It should be mentioned that enhanced plasma gastrin in response to a stable choline ester has been noted in the dog only with huge doses of the agent (Sjödin & Nilsson, 1973).

Metiamide was a generous gift from Professor J.W. Black, Smith, Kline and French Laboratories Ltd., England. The investigation was supported by grants from the Swedish Medical Research Council and the Medical Faculty of the University of Lund, Sweden.

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(Received December 16, 1974.
Revised April 12, 1975.)

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THE ROLE OF H_1 AND H_2-RECEPTORS IN THE CORONARY VASCULAR RESPONSE TO HISTAMINE OF ISOLATED PERFUSED HEARTS OF GUINEA-PIGS AND RABBITS

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1 The effects of histamine on the isolated perfused hearts of guinea-pigs and rabbits were examined. Records of contractile force, heart rate and coronary perfusion pressure were obtained.

2 Histamine exerted positive inotropic and chronotropic effects which were antagonized by burimamide and attributed to stimulation of H_2-receptors.

3 The coronary vascular response to histamine differed between guinea-pigs and rabbits. In guinea-pig hearts, three phases were apparent: (a) An initial vasodilatation preceding any effects on heart force and rate was antagonized by mepyramine and therefore mediated by histamine H_1-receptors in the coronary circulation. (b) A secondary vasoconstriction was attributed to the increased myocardial compression during the positive inotropic and chronotropic responses. (c) The final, more predominant, component was a prolonged vasodilatation probably associated with the increased metabolic activity of the heart.

4 The latter two components were abolished together with the myocardial responses by burimamide. The remaining coronary vascular response was biphasic, consisting of a vasodilatation immediately followed by vasoconstriction. Both were antagonized by mepyramine and therefore mediated by H_1-receptors.

5 The coronary vascular response of rabbit hearts was similar but no direct vasodilatation was observed and it was concluded that histamine receptors in the coronary vasculature involve only vasoconstriction.

Introduction

The stimulant effects of histamine on the heart have been shown to be antagonized by the classical antihistamines to varying degrees. Complete antagonism of the positive inotropic and chronotropic responses was claimed by Dews & Graham (1946) and Mannaioni (1960), while Bartlet (1963) and Levi & Kuye (1974) failed to show any antagonism. Others (Trendelenburg, 1960; Flacke, Atanacković, Gillis & Alper, 1967; Fantozzi, Ledda, Mannaioni, Moroni & Mugelli, 1974; McNeill & Verma, 1974a) have found only partial inhibition. This has been termed non-specific by Trendelenburg (1960) since concentrations in excess of 10^{-6}M were required, thus yielding a low pA_2 value of 5.3 compared with 9.0 obtained in the guinea-pig intestine. McNeill & Verma (1974a) found that promethazine in concentrations above 8×10^{-6}M depressed the maximum response to histamine and concluded that the antagonism was of a non-competitive nature. Since these concentrations of the antihistamine also tend to depress the rate and force of contractions (Levi & Kuye, 1974) probably by their local anaesthetic action (Trendelenburg, 1960), it is not surprising that the histamine and indeed nicotine (Trendelenburg, 1960) and noradrenaline (McNeill & Verma, 1974a) are also inhibited. The failure of these antihistamines satisfactorily to antagonize the effects of histamine on the heart and several other tissues in concentrations more appropriate to typical antihistamine activity, led to the suggestion that there are two classes of histamine receptors. Those sensitive to classical antihistamines were designated H_1-receptors (Ash & Schild, 1966). This was further substantiated by the introduction of burimamide, a selective antagonist of those effects previously unaffected by antihistamines and therefore mediated by H_2-receptors (Black, Duncan, Durant, Ganellin & Parsons, 1972). Burimamide effectively blocks the positive inotropic and chronotropic responses to histamine

(Black *et al.*, 1972, Capurro & Levi, 1973; Levi & Lee, 1974; McNeill & Verma, 1974b; Levi, Capurro & Lee, 1975).

On the coronary circulation histamine produces vasodilatation revealed as an increased coronary blood flow (Wégria, 1951; Charlier, 1961; Parratt, 1968). This response has been prevented in the guinea-pig perfused heart by the H_2-receptor antagonist metiamide, concomitantly with the antagonism of the tension and rate changes (Ercan, Bökesoy & Türker, 1974). All three responses were unaffected by mepyramine. They therefore concluded that the coronary vasodilatation in response to histamine is mediated via H_2-receptors in the coronary vasculature. However, they did not make allowance for the simultaneous blockade of the myocardial responses which may well have indirectly modified the vascular response. Flacke *et al.* (1967) also reported failure of the classical antihistamines to antagonize the increased coronary blood flow to histamine in dog heart-lung preparations. In contrast, Levi & Kuye (1974), using guinea-pig isolated hearts, have antagonized the histamine-induced increase in coronary flow by five classical H_1-antagonists. They suggest the presence of H_1-receptors in the coronary circulation. These contradictory views pose the question that both H_1- and H_2-receptors may be involved and that these authors are recording different components of the response. The present study was undertaken to examine the coronary vascular response of guinea-pig isolated hearts to histamine in order to determine whether there are several phases and the role of the simultaneous myocardial changes which were ignored by Ercan *et al.* (1974). Then the systematic use of both types of antagonist would attempt to clarify whether H_1- or H_2-receptors or both are involved in the coronary vascular response.

Finally, in view of certain reported species differences to histamine on vascular smooth muscle, an examination has also been made of the effects in rabbit hearts. For example, in the cat, rat and dog, histamine exerts vasodepressor responses through stimulation of both H_1- and H_2-receptors, whereas in the rabbit, rises in blood pressure are mediated through H_1-receptors (mepyramine-sensitive) and falls in pressure through H_2-receptors (burimamide-sensitive) (Parsons & Owen, 1973).

Methods

Guinea-pigs of either sex and weight range 450-650 g were killed by a blow on the head. The hearts were rapidly excised and transferred to a beaker containing Krebs-bicarbonate solution, where associated pericardial tissue and lung tissue were trimmed free. The cut aorta was tied into a glass cannula for retrograde perfusion of the coronary circulation by a modification (Broadley, 1970) of the Langendorff (1895) method.

A constant rate of perfusion (5 or 6 ml/min depending on animal weight) was produced with a Watson-Marlow flow inducer (MHRE/30/T, 0.8 mm tube bore). The Krebs-bicarbonate solution (composition in g/l distilled water: NaCl 6.92, KCl 0.345, $CaCl_2 2H_2O$ 0.28, $NaHCO_3$ 2.1, $MgSO_4 7H_2O$ 0.29, glucose 2.0, $NaH_2PO_4 2H_2O$ 0.16) was gassed with 5% CO_2 in O_2 before entering the flow inducer, it then passed to a warming coil (37.5° C) at the base of which was the aortic cannula. Alterations in perfusion pressure (resting level between 25 and 50 mmHg (1 mmHg $\approx$ 133 Pa), arising from changes in coronary vascular resistance were recorded on a Devices M19 polygraph by means of a pressure transducer (Consolidated Electrodynamics Type 4-327-L221) situated at a side-arm of the aortic cannula. A Condon manometer was also included in the system at this point to accommodate some degree of volume change during drug responses.

Isometric contractions of the hearts were recorded with a transducer (Ether, Type UF1, 57 g sensitivity range) attached via a pulley to a clip on the apex of the ventricles. The transducer was adjusted so that a diastolic tension of 1-2 g was applied to the hearts. The heart rate was recorded with a ratemeter (Devices, Type 2751) triggered by the signal from the tension record.

Experiments were also performed with hearts from rabbits (Norfolk White strain) of either sex and weight range 1 to 1.5 kg. These were set up identically, except that a perfusion rate of 8 ml/min was required.

Drugs were injected into the perfusion solution through the connecting rubber tubing immediately prior to entry into the warming coil. The dose volume was between 0.025 and 0.2 ml and this produced a small injection artefact which was well separated from the drug response. Antagonists were usually added to a Ringer solution reservoir.

Drugs used were: burimamide (SK & F Labs Ltd.), histamine acid phosphate (Sigma), mepyramine maleate (Anthisan, May & Baker Ltd.). All solutions were freshly prepared in 0.9% w/v NaCl solution (saline) and amounts referred to in the text are expressed as the base.

Results

Figure 1 illustrates a typical response to histamine (1 μg) of the guinea-pig perfused heart. The force

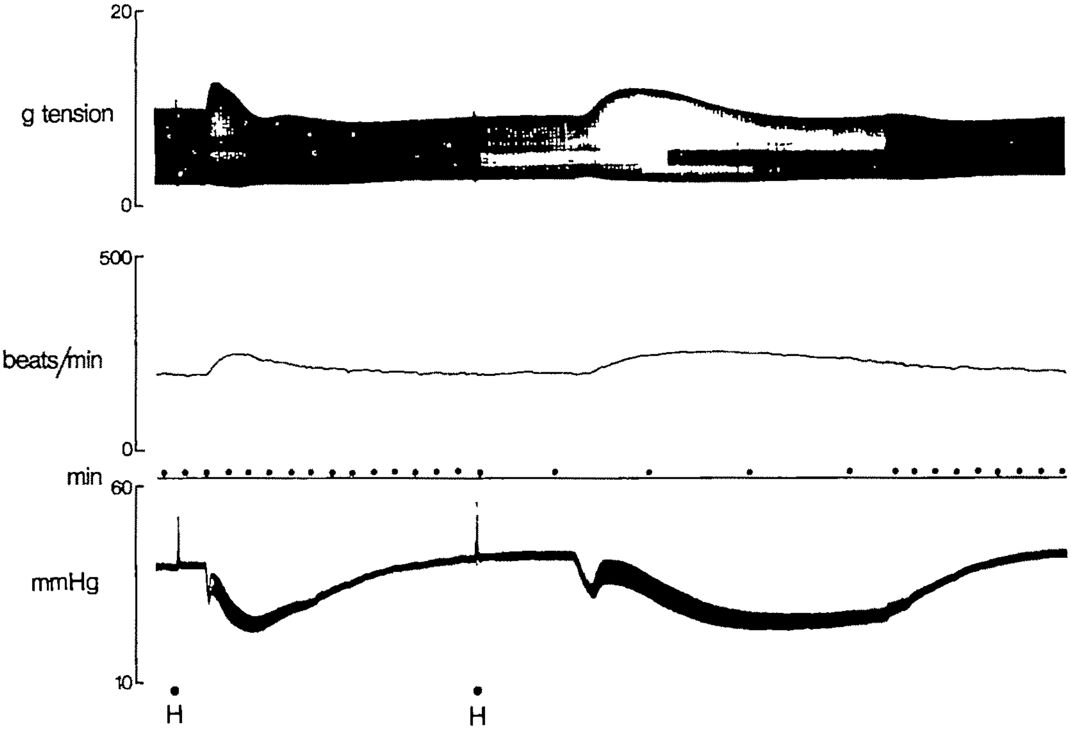

Figure 1 Guinea-pig isolated perfused heart. The effects of histamine (H, 1 μg) on the contractile force (upper record), heart rate (middle record) and coronary perfusion pressure (lower record). Administration was initially at a chart speed of 5 mm/min and repeated at 25 mm/min to separate the components of the vascular response.

and rate of contraction increased. The dose was repeated at a faster chart speed to separate three well defined phases of the coronary vascular response. These were: (a) An initial fall in perfusion pressure which preceded any effects on the force and rate of heart beat; (b) a secondary rise in pressure which commenced simultaneously with the onset of the positive inotropic and chronotropic responses. This component of the response was associated with more exaggerated excursions of the recorder stylus on either side of the mean pressure; (c) a fall in perfusion pressure which occurred while the heart was still excited, but continued after the force and rate of contractions had returned to normal. The coronary vascular response is therefore not a simple vasodilatation but consists of several components and the possible modification of each of these by the H_1- and H_2-receptor antagonists was next examined.

The initial coronary dilatation was antagonized by the inclusion of the classical antihistamine mepyramine (1 μg/ml) in the perfusion solution (Figure 2b). The increases in force and rate of contraction were unaffected and a biphasic coronary vascular response remained. This consisted of the rise in pressure coinciding with the onset of force and rate responses, followed by an unaltered predominant fall in perfusion pressure. Both these vascular responses were removed when the myocardial effects were abolished by the further introduction of the H_2-receptor antagonist burimamide (20 μg/ml) to the perfusion solution (Figure 2c).

When the order of administration of the antagonists was reversed (Figure 3), initial perfusion with burimamide (20 μg/ml) antagonized the myocardial effects of histamine, leaving a biphasic coronary vascular response (Figure 3b). The initial sharp fall in perfusion pressure was still present but was not followed by a rise in pressure; however, this was now accompanied by the myocardial effects previously observed prior to burimamide. Both of these components of the remaining response of the coronary circulation were antagonized by the addition of mepyramine (1 μg/ml) to the burimamide infusion (Figure 3c).

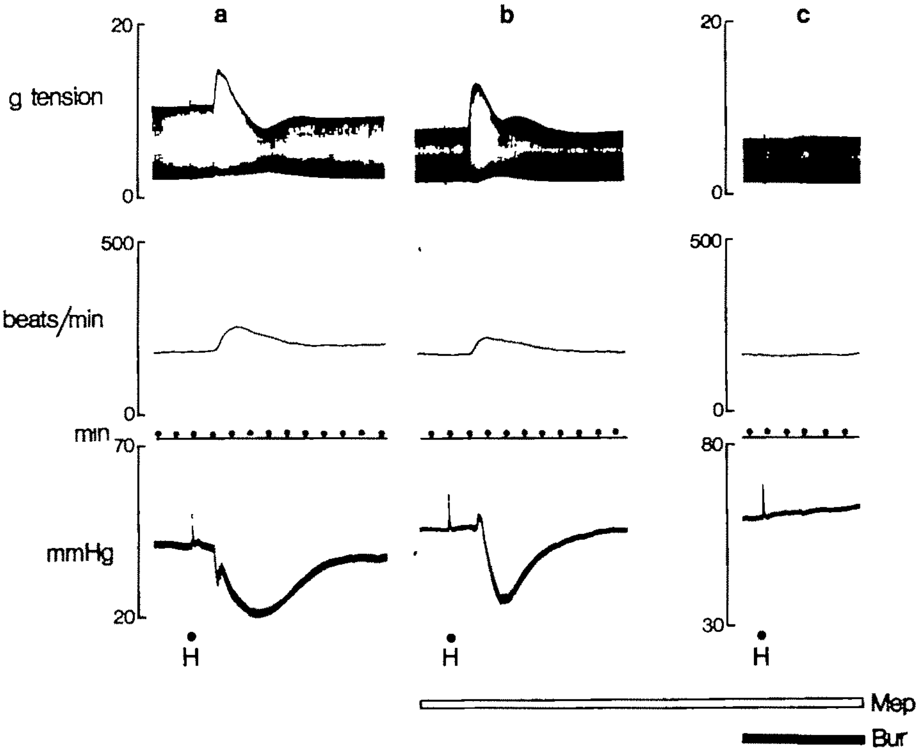

Figure 2 Guinea-pig isolated perfused heart. The effects of mepyramine alone (Mep, 1 μg/ml at open horizontal bar in (b) and combined with burimamide (Bur, 20 μg/ml) at solid horizontal bar in (c)) on the response of contractile force (upper record), heart rate (middle record) and coronary perfusion pressure (lower record) to histamine (H, 1 μg).

This figure also illustrates the rapid reversal of the burimamide blockade. Immediately after returning to a perfusion solution containing only mepyramine (Figure 3c), histamine was administered and the return of the force and rate responses began. Thereafter, the histamine dosage was repeated at regular intervals with complete restoration of the myocardial effects of histamine. Finally, the mepyramine was omitted, but even after 1 h of perfusion with this drug-free solution, the initial mepyramine-sensitive vasodilator response failed to return (Figure 3d), thus demonstrating the prolonged activity of mepyramine. The response of the coronary circulation at this point was characterized by the exaggerated excursions of the pen stylus during the rise in pressure. This differed from the rise in pressure seen before the mepyramine antagonism (Figure 3b) where only a smooth rise in pressure occurred with no change in pulse pressure. At the same time no myocardial effects were present.

Therefore two constrictor components of the coronary vascular response to histamine can be distinguished. The first is associated with a more pulsatile pressure trace and occurs concurrently with pronounced increases in force and rate on contraction. The second vasoconstriction is more delayed, has no increased pulse pressure and is present after blockade of the myocardial effects by burimamide. It is antagonized by mepyramine.

The investigation was next extended to the hearts of rabbits. Histamine (10 μg) produced positive inotropic and chronotropic responses and in the majority of preparations the coronary vascular response was biphasic, consisting of an initial rise in perfusion pressure followed by a more prolonged fall in pressure (Figure 4a). The positive chronotropic response to histamine was inhibited by burimamide (20 μg/ml), whereas the increase in tension was converted to a negative inotropic response. Histamine now caused only a vasoconstriction of the coronary circulation

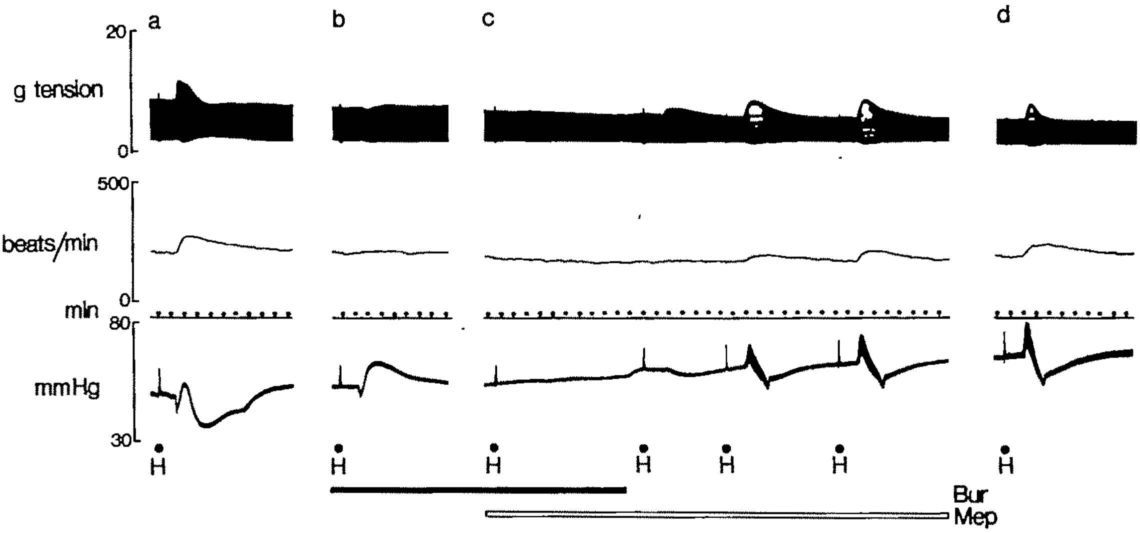

Figure 3 Guinea-pig isolated perfused heart. The effects of both addition and removal of burimamide (Bur, 20 μg/ml at solid horizontal bar) and mepyramine (Mep, 1 μg/ml at open horizontal bar) on the responses of the contractile force (upper record), heart rate (middle record) and coronary perfusion pressure (lower record) to histamine (H, 1 μg). The burimamide was omitted from the perfusion solution during (c) and in (d) histamine was repeated 1 h after perfusion with a drug-free solution.

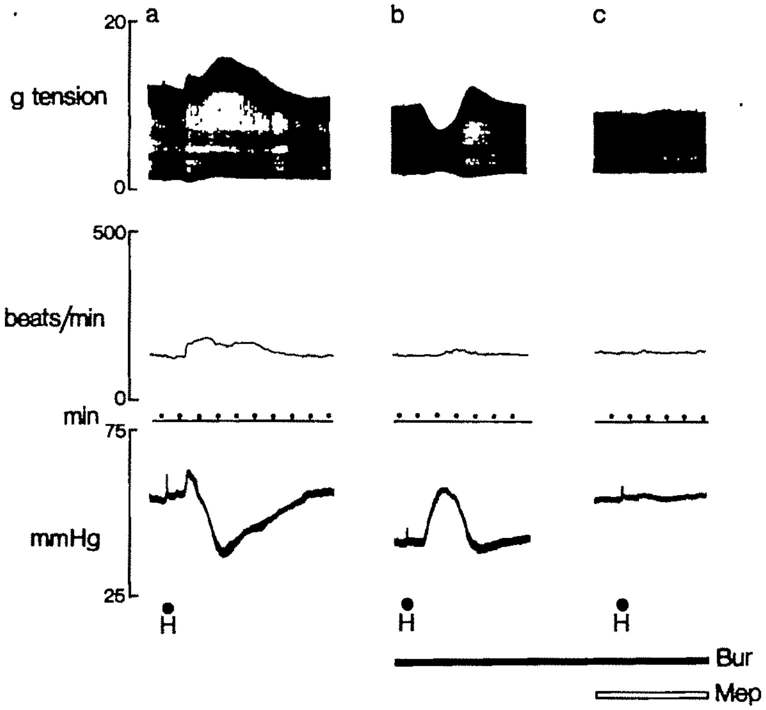

Figure 4 Rabbit isolated perfused heart. The effects of burimamide alone (Bur, 20 μg/ml at solid horizontal bar in (b)) and combined with mepyramine (Mep, 1 μg/ml at open horizontal bar in (c)) on the responses of contractile force (upper record), heart rate (middle record) and coronary perfusion pressure (lower record) to histamine (H, 10 μg).

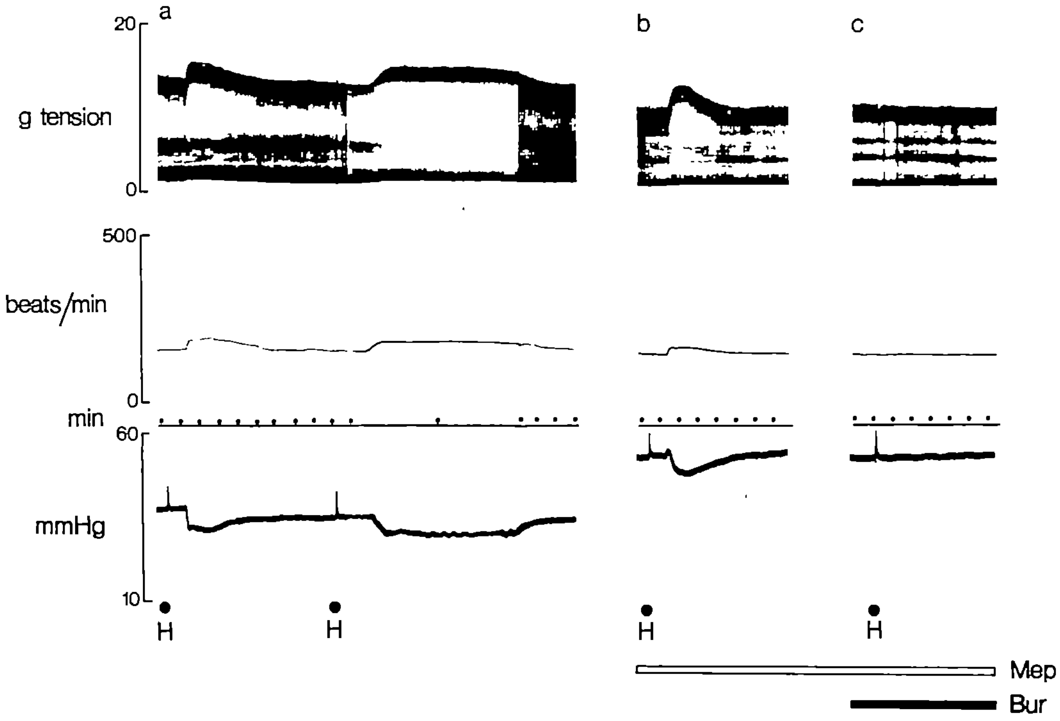

Figure 5 Rabbit isolated perfused heart. (a) Responses of the contractile force (upper record), heart rate (middle record) and coronary perfusion pressure (lower record) to histamine (H, 10 μg) at a chart speed of 5 mm/min and repeated at 25 mm/minute. (b) The effect of including mepyramine (Mep, 1 μg/ml at open horizontal bar) in the perfusion solution on the histamine responses. (c) The effect of both burimamide (Bur, 20 μg/ml at solid horizontal bar) and mepyramine on the responses to histamine.

(Figure 4b). This was abolished by the further introduction of mepyramine (1 μg/ml) as was the negative inotropic response (Figure 4c). In a few preparations there was no initial coronary vasoconstriction to histamine, instead, only a monophasic vasodilatation occurred. In these cases, a repeated dose of histamine at a faster chart speed revealed that the falls in perfusion pressure had followed the changes in force and rate of contraction (Figure 5a). This was therefore not comparable with the initial fall in pressure seen with guinea-pig hearts, which preceded any myocardial effects. Furthermore, it was not antagonized by mepyramine (Figure 5b). This coronary vascular response was abolished along with the myocardial responses by the secondary addition of burimamide (Figure 5c). Even in these rabbit hearts that exhibited solely vasodilator responses to histamine initially, only a vasoconstriction of the type seen in Figure 4b was present after the use of burimamide as the first antagonist.

The dose of histamine used in both species was submaximal, as confirmed by the preliminary construction of dose-response curves. These are plotted for guinea-pig hearts in Figure 6 before and during perfusion with burimamide (20 μg/ml). The tension and rate responses were measured as the increase to the peak effect (Figure 6a and b), and the total coronary vasodilatation was measured to the maximum fall in perfusion pressure (Figure 6c). Both the tension and rate responses were antagonized by burimamide and so too was the coronary vasodilatation. However, this coronary vascular response was divided into its component parts and the dose used throughout this study, 1 μg, was examined in more detail. The overall fall in pressure was clearly inhibited by burimamide but to a level that corresponded to the initial vasodilator component. When measured separately, this initial phase was unaltered and this fact is represented by the histograms in Figure 6d for the 1 μg dose of histamine.

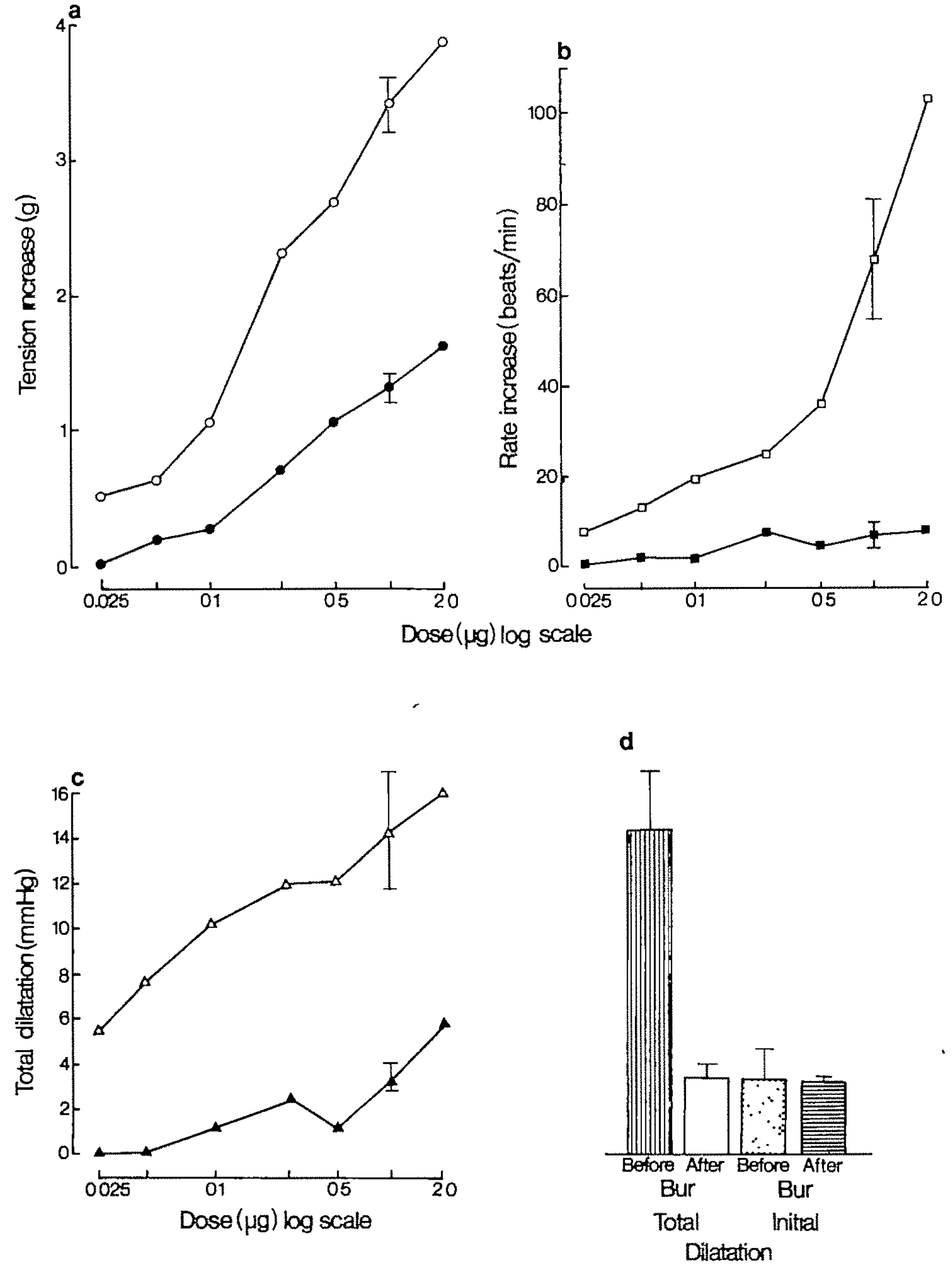

Figure 6 The effects of burimamide on the force, rate and coronary vascular responses of guinea-pig perfused hearts to histamine. Mean values were obtained from the same preparations. (a) Increase in tension in response to increasing doses of histamine before (○) and during (●) perfusion with burimamide (20 µg/ml). (b) Increase in rate in response to these doses of histamine before (□) and during (■) perfusion with burimamide (20 µg/ml). (c) Total fall in coronary perfusion pressure to the same increasing doses of histamine before (△) and during (▲) perfusion with burimamide (20 µg/ml). (d) Separation of the coronary vascular response to the 1 µg dose of histamine into the total fall in perfusion pressure before (vertical hatched column) and during (open column) perfusion with burimamide, and the initial rapid fall in perfusion pressure before (stippled column) and during (horizontal hatched column) burimamide perfusion (20 µg/ml).

Discussion

The stimulant effect of histamine upon the force and rate of cardiac contraction in both guinea-pig and rabbit hearts were not antagonized by the prototype antihistamine mepyramine. This confirms the previous observations of Trendelenburg (1960) and Bartlet (1963) that led to the classification of these effects as mediated via H_2-receptors (Black *et al.*, 1972, Ercan *et al.*, 1974; Levi & Kuye, 1974). However, these positive inotropic and chronotropic responses were antagonized by the H_2-receptor antagonist burimamide, as also shown by others (Black *et al.*, 1972; Capurro & Levi, 1973; Levi & Lee, 1974; McNeill & Verma, 1974b; Levi *et al.*, 1975). The antagonistic activity of burimamide was extremely short-lived and only persisted while the drug was present in the perfusion solution. The responses were rapidly restored to their original size on returning to a drug-free perfusion.

The overall effect of histamine on the coronary circulation *in vivo* and *in vitro* is one of vasodilatation, although this may depend upon the species (Parratt, 1968) and dose employed (Wégria, 1951; Charlier, 1961; Parratt, 1968). The predominant effect recorded here was a prolonged fall in perfusion pressure, indicative of vasodilatation. In guinea-pig perfused hearts, this vasodilatation has previously been shown to be blocked by the prototype H_1-receptor antagonists (Levi & Kuye, 1974). On the other hand, Ercan *et al.* (1974) have demonstrated blockade in the same species by the H_2-receptor antagonist metiamide along with the force and rate responses, although mepyramine was claimed to be ineffective. This conflicting evidence may well be explained by the separation here of the histamine-induced coronary vascular response of guinea-pig hearts into several well defined phases.

The first stage of the response was a fall in perfusion pressure occurring before the onset of any myocardial changes. Since this was antagonized by mepyramine, it is a direct effect on the coronary blood vessels due to vasodilatation mediated via histamine H_1-receptors. Therefore, in this respect, the results agree with those of Levi & Kuye (1974).

A secondary vasoconstrictor response commenced simultaneously with the increases in force and rate induced by histamine. It was accompanied in most preparations by exaggerated excurions of the recorder stylus and was only present when there were pronounced changes in force and rate of contraction. Therefore burimamide, in antagonizing these myocardial effects, also removed this component of the vascular response. Increases in force of contraction and tachycardia have variously been claimed to increase coronary flow indirectly by a massaging effect (Wiggers, 1954) or to restrict flow by extravascular compression of the coronary vessels by the myocardium (Sabiston & Gregg, 1957). The latter phenomenon has received most favourable support and during drug-induced positive inotropic and chronotropic responses a greater resistance to flow can be expected. For example, a similar restriction in flow has been shown to accompany the force and rate responses to adrenaline in isolated perfused hearts (Melville & Lu, 1950; Douglas, Armengol & Talesnik, 1960). It is therefore suggested that this result of increases in the force and rate of the heart also applies to histamine, which produces a secondary vasoconstriction by a compressing effect on the coronary vessels by the myocardium. Its removal by burimamide is therefore merely a consequence of the blockade of the myocardial responses.

The fall in perfusion pressure that followed was longer lasting and is recognized as the predominant coronary effect of histamine in most species both *in vivo* and *in vitro* (Wégria, 1951; Charlier, 1961; Parratt, 1968). Its antagonism by burimamide compares with the findings of Ercan *et al.* (1974) using metiamide, who therefore assumed the presence of histamine H_2-receptors in the coronary vasculature. The dose-response curves to histamine clearly demonstrate this antagonism by burimamide of the overall vasodilatation. However, closer examination reveals that it is inhibited only to the level of the initial vasodilatation of the coronary vessels, mediated via H_1-receptors.

The appearance of the secondary fall in pressure only when significant changes in force and rate of contraction are produced by histamine and the fact that its magnitude was apparently proportional to the size of the cardiac responses suggests that the two effects are closely related. The occurrence of both biphasic coronary flow changes and biphasic rate responses also led Levi (1972) to deduce that they might be interdependent. The increase in myocardial activity may lead to the release of a metabolite that exerts a vasodilator effect on the coronary vasculature, which can outlast the changes in force and rate of contraction. Increases in myocardial activity are known to lead to a relative anoxia (Berne, 1958) which elicits a strong vasodilator action on the coronary circulation (Berne, 1964). Proposed mediators of this response to anoxia are the release of adenosine (Rubio & Berne, 1969) and prostaglandins (Kent, Alexander, Pisano, Keiser & Cooper, 1973; Wennmalm, Pham-Huu-Chanh & Junstad, 1974). These metabolites may therefore also be released by a relative anoxia arising from increases in myocardial activity induced by histamine. Indeed, the overall coronary vasodilatation exerted by catecholamines has been attributed to the accompanying increased metabolic activity of the heart (Berne, 1964).

Histamine therefore exerts coronary vasodilatation by two mechanisms; a direct relaxation of coronary blood vessels mediated via H_1-receptors and a more predominant metabolically linked vasodilatation arising from the increased myocardial activity. The antagonism of the second component by burimamide is therefore a result of blockade of H_2-receptor-mediated myocardial effects and not of vascular H_2-receptors. This would contradict the conclusions of Ercan *et al.* (1974), although the existence of H_2-receptors in the coronary vasculature cannot be totally excluded by the present study.

The final component of the coronary vascular response to histamine is a vasoconstriction that is revealed after antagonism of the myocardial effects with burimamide. The myocardial stimulation normally produces vasoconstriction through extravascular compression. In addition, there is a vasoconstriction which follows the direct vasodilatation, and like the vasodilatation, is antagonized by mepyramine. It is therefore a direct effect on the coronary vasculature mediated via H_1-receptors and we can conclude that both vasoconstrictor and vasodilator effects are elicited by stimulation of H_1-receptors present in the coronary circulation of the guinea-pig.

The separation of the coronary vascular response of the guinea-pig isolated heart to histamine into four components may help to clarify the apparently anomalous results in the literature. One group of workers have antagonized the vasodilatation by mepyramine-like antihistamines (Levi & Kuye, 1974), whereas Ercan *et al.* (1974) failed to do so, but were successful with metiamide. The present study also revealed a failure to antagonize the gross vasodilatation with mepyramine, although the initial direct component was susceptible to blockade without significantly altering the overall size of the response. Close examination of the trace produced by Ercan *et al.* (1974) reveals a small inflection on the descending portion of the vasodilatation which may suggest the presence of the two vasodilator components that are clearly demonstrated in this paper; they however did not draw attention to this. The secondary direct vasoconstriction seen here only after burimamide was also sensitive to mepyramine. It is possible that removal of this component would effectively enhance the metabolically linked vasodilatation so masking any possible antagonism by mepyramine of the overall vasodilatation as claimed by Levi & Kuye (1974). However, if this were so, then the argument should apply equally to their experiments, and it does not alter the fact that the overall vasodilatation was not visibly antagonized by mepyramine. One conclusion is that different aspects of the histamine response have been recorded by the various experimental procedures. Levi & Kuye (1974) were possibly observing increases in coronary flow mediated predominantly by the direct effects on the coronary vasculature, whereas the responses in the present work and in that of Ercan *et al.* (1974) and Flacke *et al.* (1967) were predominantly indirect, resulting from the increased myocardial activity. It is also possible that the classical antihistamines used by Levi & Kuye (1974) were exerting some non-selective depressant effects both on the vasculature and on the myocardium thus reducing the metabolically-linked vasodilatation.

When the hearts of rabbits were examined, it was apparent that histamine failed to produce the initial direct vasodilatation mediated via H_1-receptors that was seen in guinea-pig hearts. Generally, biphasic coronary vascular responses were recorded, in which a constriction was followed by a more prolonged fall in pressure. Some hearts exhibited solely vasodilator responses, but these were unaffected by mepyramine and were converted to monophasic constrictor responses by burimamide. In fact all rabbit hearts produced purely constrictor responses after antagonism of the myocardial effects by burimamide. This was in turn blocked by mepyramine, indicating that it was a direct H_1-receptor effect on the coronary vessels.

At the same time as the direct vasoconstriction found in both rabbit and guinea-pig hearts during the burimamide antagonism, negative inotropic responses to histamine invariably occurred. It is unlikely that these were related either to the burimamide-resistant negative dromotropic responses that have been reported (Flacke *et al.*, 1967; Levi & Lee, 1974) or to the negative inotropic response seen *in vivo* and blocked by burimamide (Powell & Brody, 1973). In the present study it was antagonized by mepyramine at the same time as the removal of the constrictor response and is therefore probably a mechanical result of the constricted coronary vasculature.

Histamine therefore exerts only coronary vasoconstriction by interaction with H_1-receptors in the rabbit, which contrasts with the guinea-pig heart where both constriction and dilatation of coronary vessels occurs. Species differences have been reported elsewhere for other vascular beds. For example, the fall in blood pressure of the cat and dog due to histamine is mediated via both H_1- and H_2-receptors producing vasodilatation

(Parsons & Owen, 1973, Powell & Brody, 1973; Flynn & Owen, 1974); however, in the calf the vasodepressor response results from a balance between a vasodilator H_1-receptor and vasoconstrictor H_2-receptor stimulation (Eyre, 1973). The fall in blood pressure of the rabbit is the reverse, being due to a combination of H_1-pressor and H_2-depressor effects (Parsons & Owen, 1973). In the present study the rabbit also differs, since histamine exerts only H_1-receptor mediated coronary vasoconstriction. In the coronary vasculature, however, these direct effects of histamine are probably masked by the more pronounced effects of extravascular compression and metabolically induced vasodilatation resulting from the increased myocardial activity.

I am grateful to Dr R.W. Brimblecombe of SK & F Labs., Welwyn Garden City, for the generous gift of burimamide.

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(Received February 10, 1975.
Revised May 21, 1975.)

Br. J. Pharmac. (1975), 54, 523-528

DOPAMINE-INDUCED AMYLASE SECRETION FROM GUINEA-PIG SUBMANDIBULAR GLAND

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1 The effects of dopamine, 5-hydroxydopamine (5-OHDA) and noradrenaline on amylase secretion from the guinea-pig submandibular gland were investigated under *in vitro* conditions.

2 All three amines greatly enhanced amylase secretion. Blockade of dopamine β-hydroxylase did not inhibit the response to dopamine.

3 Noradrenaline and dopamine stimulated amylase release from salivary glands of reserpine-treated animals, whereas 5-OHDA had no stimulatory effect on secretion in guinea-pigs pretreated with reserpine.

4 Haloperidol completely inhibited dopamine-induced enzyme discharge, but did not affect noradrenaline-initiated secretion.

5 Apomorphine caused a slight enzyme release by itself; it diminished the dopamine secretory effect, but did not modify that of noradrenaline.

6 Pimozide and fluspirilene attenuated the dopamine-induced enzyme discharge, but compared with haloperidol they were less effective.

7 It is concluded that dopamine exerts a secretagogic action different from that of noradrenaline. The possible presence of a specific dopamine receptor in salivary glands is discussed.

Introduction

Catecholamines, by stimulating the adenylate cyclase system, are most potent inducers of enzyme secretion in salivary glands. Parasympathomimetic agents, although potent, are less effective (Schramm, 1967; Carlsöö, Danielsson, Marklund & Stigbrand, 1972). Noradrenaline-induced enzyme secretion from salivary glands is inhibited by β-adrenoceptor blocking agents but not by α-adrenoceptor blockers. Dopamine, a precursor substance in the biosynthesis of noradrenaline, is also a potent inducer of amylase and peroxidase discharge from the guinea-pig submandibular gland. However, the dopamine initiated secretion is inhibited not only by β-adrenoceptor blockers but also by α-adrenoceptor blockers (Carlsöö, Danielsson, Marklund & Stigbrand, 1974). This difference in the pattern of inhibition would seem to indicate that the action of dopamine differs from that of noradrenaline and adrenaline. A specific dopamine receptor has recently been postulated for the exocrine pancreas of the dog (Hashimoto, Satoh & Takeuchi, 1971: Furuta, Hashimoto, Iwatsuki & Takeuchi, 1973). The different effects of dopamine on the one hand and noradrenaline and adrenaline on the other hand on enzyme secretion from guinea-pig submandibular gland, could be due to a specific dopamine receptor in this gland as well.

The present study was designed to investigate in more detail the effects of dopamine on enzyme secretion from salivary glands. In an effort to establish the possible existence of a specific dopamine receptor, different drugs known to antagonize the effect of this biogenic amine were used. The experiments were performed on incubated guinea-pig submandibular gland slices.

Methods

Animal preparations

Male guinea-pigs, three months of age, weighing roughly 300 g were used for the investigation. The animals were starved overnight before being killed. In one series of experiments, nine animals were given an intraperitoneal injection of reserpine (10 mg/kg body weight) 12 h before killing them. Nine animals not given reserpine were used as controls. In all the experiments the submandibular glands of both sides were rapidly excised under sodium pentobarbitone anaesthesia. Under a stereomicroscope extraglandular fat and connective tissue were removed. In all experiments except those involving reserpine pretreatment the glands from two guinea-pigs were pooled. In the study on the effects of pretreatment with reserpine, glands from one control and one treated animal were used for each experiment. They were cut into small fragments, which were distributed among the incubating vessels. All specimens were preincubated for 30 min in 3 ml of bicarbonate buffer (pH 7.4) supplemented with pyruvate, glutamate and fumarate (Krebs, 1950) and also containing albumin (5 mg/ml) and glucose (0.6 mg/ml). After preincubation the medium was replaced by 3 ml of fresh incubation buffer containing different secretagogues and test-substances. Control incubations without additives were included in each experiment. The dopamine receptor blocking agents were present during both the preincubation and the incubation periods.

Preincubation as well as incubation was carried out at 37° C under continuous equilibration of the medium with 95% O_2 and 5% CO_2 in a metabolic shaker (Danielsson, 1974). After 60 min of incubation the specimens were removed by filtration through a nylon net and were homogenized in 3 ml of 50 mM phosphate buffer (pH 6.9), using an Ultra-Turrax homogenizer (Janke und Kunkel K.G., Staufen, Germany) run at a speed of 9,600 rev/min for 45-60 s at 4° C. The homogenates were centrifuged at about 3000 *g* for 5 minutes. The amylase activities of the crude homogenate and supernatant did not differ significantly. Both incubation media and supernatants were assayed for amylase.

Amylase assay

Samples of tissue extracts (supernatants) or incubation media were appropriately diluted with 50 mM phosphate buffer and assayed for amylase by a micro-modification of the 3,5-dinitrosalicylate (DNS) method with 2% soluble starch as substrate (Danielsson, 1974). One unit of amylase was defined as the activity liberating reducing groups corresponding to 1 μmol of maltose monohydrate per min at 25° C. The amylase release was expressed both as units/g tissue wet weight recorded in the medium and as percentage of the total amylase activity in tissue and medium.

Chemicals and drugs

Soluble starch and 3,5-dinitrosalicylic acid were obtained from E. Merck A.G., Darmstadt, Germany. All chemicals were of analytical grade. (−)-Noradrenaline bitartrate was purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks. 3-Hydroxytyramine hydrochloride (dopamine) was from Sigma Chemical Co., St. Louis, Mo., U.S.A. Reserpine (Serpasil) was from Ciba-Geigy A.G., Basel, Switzerland and apomorphine hydrochloride from Sandoz A.G., Basel, Switzerland. Sodium diethyl-dithiocarbamate (DDTC) was from Fluka A.G., Buchs, Switzerland, and sodium pentobarbitone (Mebumal) from ACO, Sweden. Haloperidol, fluspirilene and pimozide were kindly donated by Janssen Division, Beerse, Belgium through A.B. Leo, Helsingborg, Sweden. 5-Hydroxydopamine was a gift from A.B. Hässle, Mölndal, Sweden.

Statistical evaluation

The statistical probability that the effect of an additive to the incubation medium was a chance effect, was ruled out from the mean differences between test and control incubations in a series of identical but separate experiments.

Results

Dopamine and noradrenaline elicited amylase secretion of about equal magnitude from guinea-pig submandibular gland slices (Table 1). DDTC, a dopamine-β-hydroxylase inhibitor, neither influenced basal enzyme release nor the secretory response to dopamine or noradrenaline (Table 1). L-DOPA (10^{-4} M) the precursor of dopamine, was found to be without significant stimulatory effect on amylase secretion (% amylase release 3.9 ± 0.64 after treatment as compared with 6.1 ± 2 98 in controls; *n* = 4).

To investigate whether dopamine exerts a direct action on the acinar cells or an indirect one by liberating noradrenaline from sympathetic nerve endings, guinea-pigs were pretreated with reserpine to deplete the endogenous stores of noradrenaline. The submandibular glands of these animals were then incubated with the secretagogues listed in Table 2. The results were compared to those obtained from non-reserpine-treated control animals (Table 2). Injection of reserpine 12 h before the *in vitro* incubations, resulted in an increase in total amylase content of the glands as compared to the glands of non-reserpine-treated animals (Table 2). Amylase secretion from submandibular glands of control animals was enhanced by dopamine, 5-hydroxydopamine (5-OHDA) and noradrenaline. 5-OHDA was without secretory effect on reserpine-treated animals, whereas dopamine and noradrenaline were still most effective secretagogues.

In Table 3 apomorphine and three neuroleptic drugs (haloperidol, pimozide and fluspirilene), which interact with dopamine receptors in the CNS, were tested on dopamine- and noradrenaline-induced amylase secretion. Apomorphine itself, displayed a slight statistically significant, enhancement of the enzyme release. On the other hand haloperidol (a butyrophenone), pimozide and fluspirilene (diphenylbutylamines) did not influence the basal secretion. Dopamine-stimulated enzyme release was antagonized by all these compounds. Haloperidol was the most efficient inhibitor and reduced amylase secretion almost to the basal level. Apomorphine, haloperidol and fluspirilene did not affect the noradrenaline-initiated amylase discharge. On the other hand, pimozide, besides attenuating amylase secretion induced by dopamine, also reduced the amylase discharge caused by noradrenaline.

Table 1 Effect of sodium diethyl-dithiocarbamate (DDTC) on noradrenaline- and dopamine-induced amylase secretion

Test-substance	n	*Amylase activity in medium*	*Amylase activity in homogenate*	*% amylase release*
Control	5	34 ± 16	414 ± 125	7.1 ± 1.47
DDTC (2 x 10^{-4} M)	5	25 ± 12	417 ± 84	5.0 ± 1.43
Noradrenaline (10^{-5} M)	5	273 ± 38	447 ± 130	40.9 ± 3.58
Noradrenaline (10^{-5} M) + DDTC (2 x 10^{-4} M)	5	210 ± 54	356 ± 74	36.7 ± 2.11
Dopamine (10^{-4}M)	5	211 ± 25	385 ± 148	41.9 ± 8.06
Dopamine (10^{-4} M) + DDTC (2 x 10^{-4} M)	5	214 ± 21	326 ± 88	43.8 ± 7.03

After a preincubation period of 30 min at 37°C the specimens were incubated in a supplemented Krebs-Ringer bicarbonate buffer for 60 min with listed concentrations of noradrenaline and dopamine The dopamine-β-hydroxylase inhibitor (DDTC; 2 x 10^{-4} M) was present during both preincubation and incubation periods. Amylase activity is expressed as u/g tissue wet weight and amylase release as % of the total amylase activity in tissue and medium. Mean values ± s.e. for indicated number of experiments.

Table 2 Effects of dopamine, 5-hydroxydopamine and noradrenaline on amylase secretion in reserpine-treated guinea-pigs

Secretagogue	n	*Amylase activity in medium*	*Amylase activity in homogenate*	*% amylase release*
Non-pretreated animals				
Control	9	46 ± 4	466 ± 47	9.8 ± 1.36
Dopamine (10^{-4} M)	6	263 ± 36**	406 ± 28	38.6 ± 3.05***
5-Hydroxydopamine (10^{-4} M)	6	135 ± 24*	407 ± 63	25.5 ± 3.19**
Noradrenaline (10^{-5} M)	8	286 ± 26***	408 ± 42	41.4 ± 3.32***
Reserpine-pretreated animals				
Control	9	56 ± 4	838 ± 95	6.6 ± 0.61
Dopamine (10^{-4} M)	6	237 ± 36**	810 ± 110	22.6 ± 1.19***
5-Hydroxydopamine (10^{-4} M)	6	60 ± 9	752 ± 92	7.8 ± 1.34
Noradrenaline (10^{-5} M)	8	420 ± 53***	814 ± 59	33.4 ± 2.27***

Nine guinea-pigs received an injection of reserpine (10 mg/kg body weight, i.p.) and nine control animals the same volume of 0.9% w/v NaCl solution 12 h before being killed. Pieces of submandibular glands from one reserpine-treated and one control animal were incubated simultaneously for 60 min at 37°C in a supplemented Krebs-Ringer bicarbonate buffer with listed concentrations of secretagogues. Amylase activity is expressed as u/g tissue wet weight and amylase release as % of the total amylase activity in tissue and medium. Mean values ± s.e. for listed number of observations.

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

Table 3 Effects of dopamine receptor blocking agents on noradrenaline- and dopamine-induced amylase secretion

	% amylase release			
Normal Control	7.8 ± 1.44 (7)	6.3 ± 2.50 (6)	5.3 ± 1.48 (8)	4.9 ± 0.53 (6)
Noradrenaline (10^{-5} M)	43.7 ± 3.85 (7)	34.9 ± 2.44 (6)	33.6 ± 1.27 (8)	36.8 ± 2.24 (6)
Dopamine (10^{-4} M)	46.8 ± 1.99 (9)	31.8 ± 3.17 (6)	32.0 ± 1.52 (7)	33.7 ± 1.56 (6)
Inhibitors	*Apomorphine (2×10^{-4} M)*	*Haloperidol (2×10^{-4} M)*	*Pimozide (2×10^{-4} M)*	*Fluspirilene (2×10^{-4} M)*
	% amylase release			
Inhibitor alone	12.8 ± 0.91** (7)	8.2 ± 2.56 (4)	4.4 ± 0.75 (5)	11.1 ± 3.35 (4)
Noradrenaline + inhibitor	38.3 ± 1.65 (7)	31.0 ± 1.47 (6)	26.5 ± 2.61* (8)	30.9 ± 2.85 (6)
Dopamine + inhibitor	32.6 ± 2.09** (9)	12.1 ± 2.81*** (6)	21.6 ± 2.17*** (7)	20.2 ± 3.46* (6)

After a preincubation period of 30 min at 37° C the specimens were incubated in a supplemented Krebs-Ringer bicarbonate buffer for 60 min with 2×10^{-4} M apomorphine, haloperidol, pimozide or fluspirilene. The dopamine receptor blockers were present during both preincubation and incubation periods. Amylase release is expressed as % of the total amylase activity in tissue and medium. Mean values (%) ± s.e. for indicated number of observations (*n* within brackets).
*** $P < 0.001$, ** $P < 0.01$; * $P < 0.05$

Discussion

L-Dihydroxyphenylalanine (L-DOPA) and 3-hydroxytyramine (dopamine) are biochemical precursors in the biosynthesis of noradrenaline. However, it has been suggested that these precursor substances may have important physiological functions of their own. The presence of a specific dopamine receptor in the CNS is well established (e.g. Hornykiewicz, 1966), and such a receptor has also been suggested for the renal and mesenteric vascular beds (Yeh, McNay & Goldberg, 1969). Dopamine greatly enhances amylase and peroxidase secretion from incubated guinea-pig submandibular gland with a maximal effect at a concentration of 10^{-4} M (Carlsöö *et al.*, 1974). Dopamine at this concentration is about as potent as noradrenaline in inducing amylase release. This effect of dopamine is inhibited by both propranolol and phenoxybenzamine (β- and α-adrenoceptor blocking agents respectively) (Carlsöö *et al.*, 1974) whereas noradrenaline action is exclusively inhibited by β-blockers. This difference in action between dopamine and noradrenaline raises the question of the possible existence of a specific dopamine receptor in salivary glands.

Dopamine does not seem to exert its effect by being converted to noradrenaline, since the presence in the incubation medium of a dopamine-β-hydroxylase inhibitor, DDTC (Molinoff & Axelrod, 1971; Tjälve, 1971) did not influence the dopamine-induced amylase release.

A 'false' neurotransmitter substance, 5-OHDA is taken up by autonomic nerve endings and replaces the physiological transmitter(s) (Thoenen & Tranzer, 1971). This amine was found to stimulate amylase secretion in the present study. Its secretagogic action does not seem to be due to a direct effect on the acinar cells, as glands from reserpine-treated animals are not stimulated. However, dopamine and noradrenaline induce amylase secretion from the submandibular gland irrespective of preceding reserpine-treatment. The percentage of amylase release in response to dopamine and noradrenaline is somewhat lower in the reserpine-treated group as compared to controls. This is partly explained by the increased total amylase content of the glands after reserpine treatment. The experiments indicate that 5-OHDA acts by liberating endogenous stores of noradrenaline, whereas dopamine seems to exert its main effect directly on the acinar cells.

Apomorphine, a known agonist for dopamine receptors in the CNS (Ernst, 1967; Andén, Rubenson, Fuxe & Hökfelt, 1967), slightly increases amylase discharge from the submandibular gland. This effect, however, is not of the same magnitude as that obtained with dopamine. On the other hand, dopamine-induced secretion is reduced by roughly 30% in the presence of apomorphine, whereas noradrenaline-stimulated release is unaffected. Some neuroleptic drugs seem to act mainly by blocking the CNS dopamine receptors (e.g. Andén, Butcher, Corrodi, Fuxe & Ungerstedt, 1970). Haloperidol inhibits dopamine-induced renal, mesenteric and coronary vasodilatation (see e.g. Goldberg, 1972). In our experiments, haloperidol almost completely inhibited the dopamine-initiated amylase discharge without affecting secretion induced by noradrenaline.

Two new potent neuroleptic drugs, pimozide and fluspirilene, which interact with the dopamine receptors of the CNS (Janssen, Niemegeers, Schellekens, Dresse, Lenaerts, Pinchard, Schaper, van Nueten & Verbruggen, 1968; Andén *et al.*, 1970; Ojeda, Harms & McCann, 1974) were also studied with respect to their effects on dopamine-stimulated secretion. Neither drug had any effect on its own on secretion but both inhibited amylase release caused by dopamine. They were less effective inhibitors than haloperidol. Furthermore, pimozide also reduced amylase release caused by noradrenaline, whereas fluspirilene did not significantly alter that secretory response.

Salivary gland activity in response to dopamine differs from that in response to noradrenaline in that it can be inhibited by both α- and β-adrenoceptor blocking agents, whereas the latter is inhibited solely by agents which block the β-adrenoceptor (Carlsöö *et al.*, 1974). In addition, the action of dopamine but not that of noradrenaline is inhibited by haloperidol, fluspirilene and apomorphine. Though the pattern of blockade is not identical with the pattern in the CNS the characteristics of the salivary gland's response to dopamine seem to be sufficiently different from noradrenaline to postulate a specific receptor for dopamine in the gland. However, there is as yet, no evidence of the presence of dopaminergic neurones in these glands.

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Br. J. Pharmac. (1975), 54, 529-539

A QUANTITATIVE MICROIONTOPHORETIC ANALYSIS OF THE RESPONSES OF CENTRAL NEURONES TO NORADRENALINE: INTERACTIONS WITH COBALT, MANGANESE, VERAPAMIL AND DICHLOROISOPRENALINE

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1 A new experimental procedure has been devised for the study of pharmacological antagonism in the central nervous system using automated microiontophoresis to deliver pulses of agonists and computer-generated histograms to quantify the neuronal response. The system allows study of potential antagonists having direct depressant effects and also of neurones with irregular or slow discharge rates.

2 The histogram analysis reveals the necessity for regular, periodic delivery of agonists during the assessment of agonist-antagonist interactions. Without regular repetitive delivery, many agonists, such as noradrenaline, exhibit an apparent but artifactual decrease in inhibitory potency after an interruption of agonist pulses. Examples of this phenomenon are shown, using cerebellar Punkinje cells and cerebral cortical neurones in rats anaesthetized with halothane.

3 Preliminary results with these computer-generated drug response histograms revealed manganese, cobalt, and verapamil to be generally ineffective as antagonists of noradrenaline, despite their direct depressant effects.

4 Conversely, dichloroisoprenaline (DCI), a β-adrenoceptor antagonist, was effective in blocking noradrenaline-induced depressions of firing in the cerebral cortex at doses which caused over 50% decrease in spontaneous discharge.

Introduction

Direct demonstration of responses to noradrenaline in central neurones and their antagonism has been made possible by application of drugs directly to the neurones by microiontophoresis. Design of experiments and analysis of the data has been complicated, however, by the many biological and technical difficulties inherent in this technique (Bloom, 1974). The present study examines three problems in the testing of antagonists of noradrenaline (NA) and demonstrates some possible solutions.

The first problem is the establishment of reproducible NA effects, so that the absence of such effects during the application of another substance can properly be interpreted as an antagonism. Clarke, Hill & Simmonds (1973) and Bradshaw, Roberts & Szabadi (1973a) showed that iontophoretic release of radio-labelled substances *in vitro* is affected by the strength and duration of previous retention currents. Work from these same laboratories has shown that the size of NA (Bradshaw, Szabadi & Roberts, 1973b) and γ-aminobutyric acid (GABA)-induced (Hill, Simmonds & Straughan, 1973) depressions in central neurones is a function of the magnitude and duration of the retaining currents. In confirmation of their findings, we report here that regular applications of uniform NA pulses are necessary for reproducible inhibitions and that interruption of these periodic applications alone is sufficient to decrease the apparent inhibitory potency of NA when the pulses are resumed. This effect would cause an apparent, but artifactual antagonism if NA pulses were interrupted during application of an antagonist.

A second problem is the evaluation of potential antagonism by an agent having additional direct depressant effects on activity. When spontaneous discharge rate drops to levels of less than one per second, it may not be possible to judge NA efficacy, and hence antagonism, by simple visual inspection of ratemeter records or spike trains. Computer-generated drug response histograms, used first by Gottesfeld, Kelly & Renaud (1972) to quantitate GABA effects, allow the technique of peri-stimulus histograms to be applied to the NA responses of these slowly discharging cells. Because the histogram sums spikes recorded during multiple, repeated trials of NA, quantitative comparisons of adrenergic inhibition can be made before, during, and after an antagonist trial. We show here how several reported adrenoceptor antagonists can be tested by this method.

Neurones which fire irregularly, such as cerebral cortical neurones in anaesthetized animals, present a third problem similar to that produced by depressant antagonists. The random bursts and long pauses characteristic of the control discharge pattern of many of these cells make it difficult to evaluate inhibition. The drug response histogram emphasizes drug related changes in firing, while unrelated bursts and pauses are randomized.

Methods

Ten male Sprague-Dawley rats, weight 200-250 gm, were used in these experiments. The animals were anaesthetized with 0.5-0.75% halothane, intubated, and allowed to breathe spontaneously. Body temperature was maintained at 35-36°C. After cisternal drainage, the skull was removed over the posterior cerebellar vermis or one of the cerebral hemispheres, posterior to the bregma. The dura was excised under a drip of warm Earle's balanced salt solution (Grand Island Biological Co.) after which 2% agar in saline was applied to the surface of the brain.

Five barrel micropipettes were prepared as described previously (Salmoiraghi & Bloom, 1964). The barrels were filled initially by boiling in distilled water, which was aspirated subsequently and replaced by drug or electrolyte. After centrifugation to accelerate drug movement into the tip, the pipettes were broken to 5-7 μm diameter under microscopic observation, a size normally used during physiological experiments. Occasionally, larger tips (22-25 μm) were produced. Pipettes with the larger tips were used to control for the possibility that the drug-time interactions noted below were a function of only a certain range of tip size. Tips larger than 25 μm could not be studied because of difficulty in recording from neurones. Resistance of all barrels before insertion into the brain was less than 200 MΩ. The centre barrel, used for recording, was filled with 5 M NaCl and generally had a resistance of 1.5-3 MΩ. Three of the four side barrels were filled with drugs; the fourth contained 3 M NaCl for current controls and to balance drug ejection and holding currents, as noted below. Holding currents of −15 nA were used whenever drugs were not ejected. Drug solutions used were: 0.5 M (−)-noradrenaline HCl, pH 4.5 (NA, Aldrich); 1.0 M γ-aminobutyric acid, pH 4.0 (GABA, Calbiochem.): 0.1 M manganese chloride (Mn), pH 5.3; 0.2 M cobalt chloride (Co), 0.06 M verapamil hydrochloride in 165 mM NaCl, pH 5 2 (a gift of Dr John Daly, NIAMD), 0.5 M dichlorophenylisopropylaminoethanol (DCI, Aldrich).

Purkinje cells recorded extracellularly in the cerebellar cortex were identified by their characteristic climbing fibre and simple spike discharge (Eccles, Ito & Szentagothai, 1967). Cells with regular discharge rates between 20 and 50 Hz were considered to be uninjured and suitable for study (Murphy & Sabah, 1970). Neurones encountered in penetrations in the parietal cerebral cortex were noted to fire more slowly and irregularly. Cortical neurones chosen for study had uniform action potential amplitudes and no obvious injury bursts.

A crystal clock-regulated stimulator (Digitimer, Devices Ltd.) controlled the iontophoresis circuit (Geller & Woodward, 1972) so that constant current pulses of uniform duration and magnitude could be passed at equal intervals through agonist barrels. An operational amplifier balancing circuit was employed in conjuction with the 3 M NaCl barrel to eject a current equal and opposite to the sum of the currents in the other three barrels at any instant (Geller & Woodward, 1972).

Extracellular action potentials were amplified by conventional means, monitored on an oscilloscope and converted to uniform voltage pulses by a window discriminator. The pulses were fed to a ratemeter which integrated them over one second intervals. The ratemeter output and the iontophoresis currents were displayed together on a polygraph.

The window discriminator output was also led to a digital computer (PDP-12, Digital Equipment Corp.) to generate an on-line drug response histogram, which summed the unit responses during several agonist pulses. Average agonist responses could thereby be computed before, during and at intervals after antagonist application. The computer was used to quantitate the agonist responses by determining the average counts per address during the response period and comparing it with control periods, expressing the difference as percent inhibition. Two control periods, one before and one after the drug ejection, were arbitrarily selected. Counts per bin were calculated and required to be within 10% of each other as criteria for stability, i.e. constancy of neuronal discharge rate. The period of drug response was selected to begin at the particular bin where counts fell significantly below baseline and terminate at the bin where counts re-approached the baseline. Once the bins corresponding to the onset and termination of drug response were chosen in the control histogram, the identical epoch was used for comparison of percent changes in all subsequent

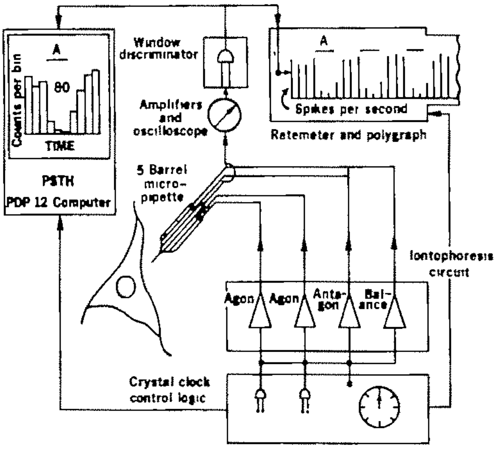

Figure 1 Summary diagram of the experimental apparatus. A logic circuit timed by a crystal clock sends control voltage pulses to an iontophoresis circuit employing operational amplifiers (lower right). The three constant current outputs apply agonists (Agon) and antagonists (Antagon) through three barrels of the five barreled micropipette (centre). Zero net iontophoretic current is maintained through an automatic balancing circuit and a fourth barrel. The neuronal action potentials are recorded extracellularly by the fifth barrel. Action potentials are amplified and converted into constant voltage pulses by a window discriminator. These pulses are either integrated over 1 s intervals by a ratemeter and displayed on a polygraph or led to a PDP-12 computer. Using the PSTH programme, the computer sums several drug responses into a histogram, as shown for agonist A. Timing pulses are provided from the clock logic. The computer calculates the percent inhibition caused by the agonist (see Methods). Responses to agonists can thus be computed before, during, and after iontophoresis of an antagonist.

histograms. The computer programme (PSTH, Woodward, 1972) uses a double precision, 24 bit clock subroutine, so that the sweep can be extended to accommodate the 'slow' responses seen with monoamine iontophoresis while maintaining clock sampling accuracy (1 KHz).

Stable agonist responses were obtained from each cell before recording the first control histogram. Particularly with NA, the response to a pulse of a given current increases markedly with the first few pulses. Residual effects of this 'warm up' phenomenon may be seen as long as 30 min after the beginning of a continuous series of NA pulses. Each antagonist was tested at least twice per cell, with control, antagonist and recovery histograms computed each time. The number of sweeps was adjusted to provide approximately equal numbers of counts in the control addresses of each histogram. Thus, more sweeps were required in histograms recorded while neuronal firing was slowed by an antagonist with direct depressant effects. Generally, cells in this study were held for 1 to 2 h and a regular cycle of agonist pulses was maintained throughout. Data from an experiment was not accepted if the agonist response failed to show recovery from antagonism. To summarize the data in tabular form, a blockade was arbitrarily defined as a reproducible reduction of at least 40% of the agonist response. A diagram of the experimental apparatus is shown in Figure 1.

Results

Timing of agonist pulses

Iontophoresis of NA resulted in inhibition of nearly all Purkinje cells and cerebral cortical neurones observed (Hoffer, Siggins & Bloom, 1971; Stone, 1973). A 2 to 5 min 'warm up' period was generally found, during which the efficacy of inhibition increased markedly without change in iontophoresis parameters. In many cases an initial NA pulse would be almost completely ineffective, but inhibition would increase consistently with the next 5 to 10 pulses. Further increases of 5 to 10% usually occurred over the next 30 min with continual periodic NA application. When a particular interval between pulses was lengthened (Figure 2), a marked decrease in the efficacy of inhibition was noted in the subsequent pulses. Interruptions of as little as 1 or 2 min markedly decreased NA evoked inhibition. Recovery of inhibition was usually not seen until at least the fourth pulse after the interruption.

Experiments testing the effect of interruption of regular, periodic NA applications were performed on 11 cells. The time for recovery of the response to its initial value was directly proportional to the length of interruption ($P < 0.01$, Figure 3B). No difference was noted between cerebral cortical neurones and Purkinje cells. The percentage decrease in inhibition was determined by calculations from drug response histograms. Responses to three pulses of NA were summed from before and after the interruption and the percent inhibition obtained in the two histograms was compared. Most neurones, recorded with pipettes of overall tip diameters of 5-7 μm, showed a 70-95% decrement in inhibition (Figure 3A). The pipettes which did not show as marked an effect generally had very large tip diameters (25 μm), but even these showed decrements of 25-50%. Because three pulses were summed to form the histogram, the magnitude of the decrease in inhibition is

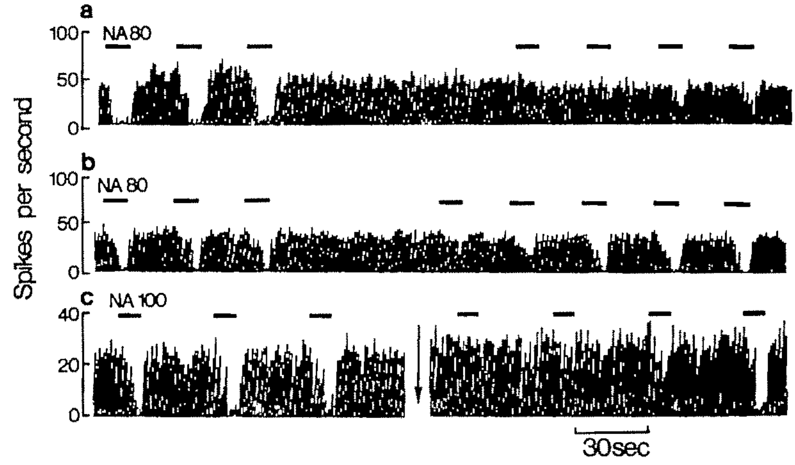

Figure 2 Effects of prolonging interpulse time on the response of Purkinje cells to iontophoresis of NA. (a) and (b) represent continuous records from one cell. (c) shows a different cell in which the arrow indicates a 2 min pause before resuming pulses. In this and all subsequent ratemeter records, the ordinate shows spikes/second. The letters and numbers over the lines indicate the drug and its ejection current (nA). The length of the lines shows the duration of drug ejection. Note the profound reversible decrement in response produced by pause of several minutes between 10 s NA pulses given at 40 s intervals.

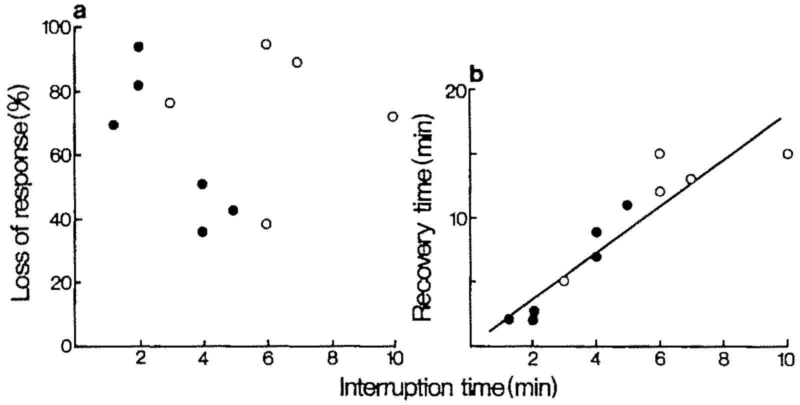

Figure 3 Effects of interruption of NA pulses on NA-induced inhibition. (a) Degree of loss of inhibition of the neuron to NA pulses as a function of pulse interruption time, calculated as the difference between NA response histograms which summed the three pulses before and the three pulses after the interruption. (b) Time for recovery of inhibitory NA responses to at least 80% of control as a function of pulse interruption time. Note linear relationship ($P < 0.01$). Most cells were given a 15 s NA pulse repeated every 60 seconds. ● Purkinje cell. ○ Cortical neuron.

under-estimated, since there was some increase in efficacy in successive pulses after the interruption. The decrease in inhibition assessed in this way was not linearly related to the length of interruption (Figure 3A).

To test whether this loss of response depended solely on the release of drug from the micropipette or whether biological factors, such as reuptake or a change in the sensitivity of the membrane, might also be important, NA was applied alternately from two barrels of the same pipette in some experiments. When the second pulse was interrupted, as shown by the drug response histograms in Figure 4, the effectiveness of the first pulse was

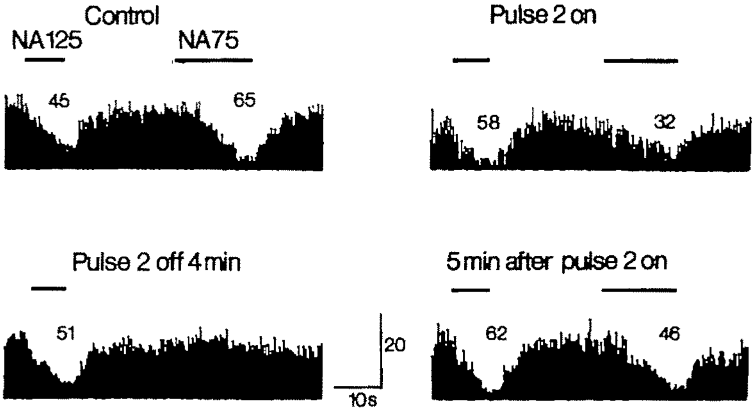

Figure 4 Effects of interruption of NA pulses on inhibition of Purkinje cell spike activity. Four drug responses histograms are illustrated, each with a total sweep time of 50 seconds. Two pulses of NA were applied through different barrels during each sweep at the times indicated by the horizontal bars in the control histogram (upper left). Cessation of the second pulse does not reduce the efficacy of the first pulse (lower left). However, the second pulse is much less effective when resumed after a 4 min interruption (upper right). Recovery is still not complete 5 min later (lower right). The continued improvement in the control response was seen frequently with periodic application of NA. Numbers below the bar on this and subsequent histograms are the % inhibition of spontaneous activity. Calibration bars are: ordinate 20 counts per address and abscissa 10 seconds.

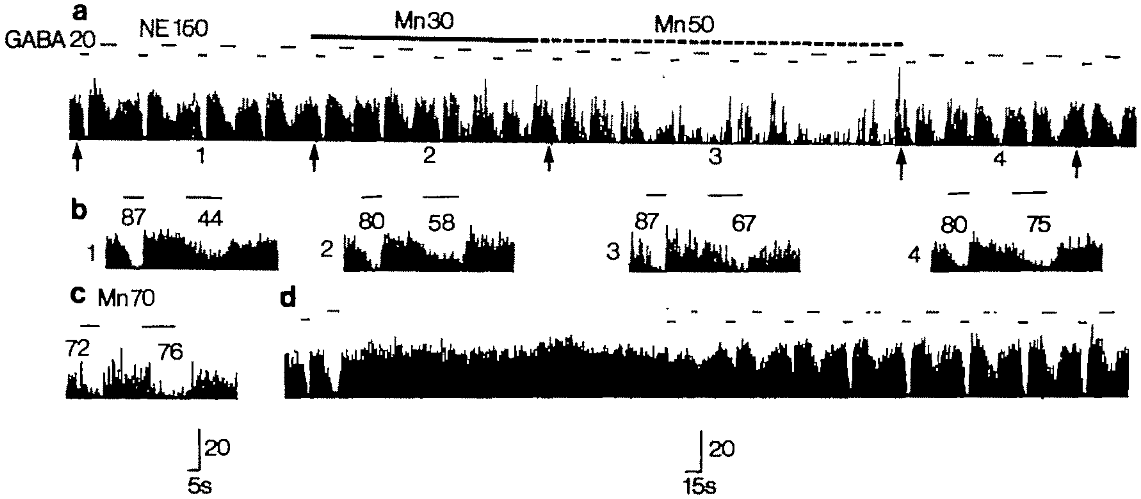

Figure 5 Ratemeter and drug response histograms showing effect of manganese (Mn) and interruption of agonist pulses on inhibitory responses to GABA (solid line) and NA (dotted line). (a), (b), (c) and (d) are all from the same Purkinje cell, recorded over a period of 6 hours. (a) shows the continuous ratemeter record of the effects of periodic application of GABA and NA. The numbers and arrows beneath the record show the regions used to construct the associated drug response histograms in (b). Note that despite the large reduction of spontaneous activity produced by 50 nA of manganese, there is no decrement in noradrenaline-induced inhibition during (b3) or after (b4) manganese. An even-larger dose of 70 nA of manganese (c) reduced spontaneous discharge rate to 0.3 spikes/s but did not reduce NA-induced inhibition. (d) shows the differential effect of pulse interruption. At the resumption of pulsing, both GABA and NA-induced inhibition are diminished. However, the GABA response recovers by the 4th cycle whereas the NA inhibition is still only 50% recovered by the 8th cycle. The calibrations bars under (c) and (d) are for histogram (counts per address) and ratemeter (spikes/s) records, respectively.

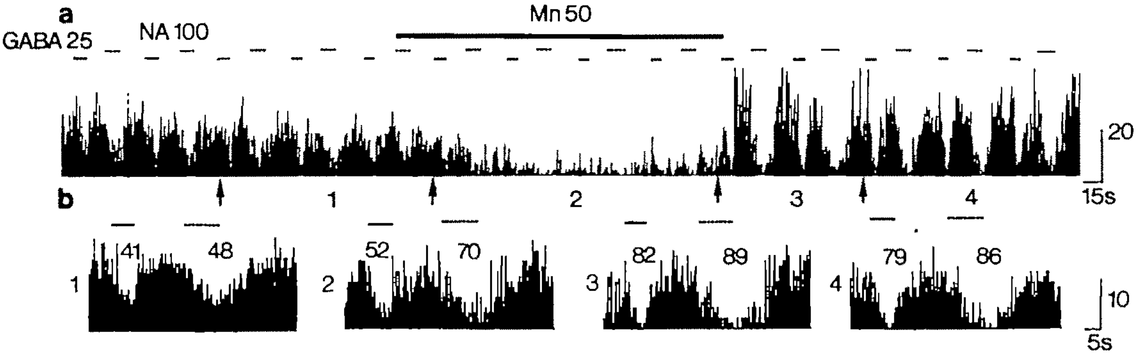

Figure 6 Effects of manganese on NA and GABA-induced inhibitions in a cerebellar Purkinje cell Continuous ratemeter recording shown in (a) and histograms in (b) Periodic application of GABA 25 nA (solid line) and NA 100 nA (dotted line) produced approximately equal inhibition. Note the profound depressant effect of manganese 50 nA (solid bar). Numbers and arrows beneath (a) show the regions used to construct associated histograms in (b). Note that there is no decrement in either NA- or GABA-induced inhibition during (b2) or after (b3, b4) this dose of manganese. Calibration bars at right in (a) are for ratemeter (spikes/s), and in (b), are for histograms (counts per address).

not diminished. When the second pulse resumed, it had a decrease in efficiency similar to that shown in the examples of Figure 2. Note that the first barrel shows continued slight improvement, presumably reflecting a continuing, residual 'warm up'.

Although the effect of pulse interruption was more pronounced with NA, a similar decrease in effectiveness was noted when GABA pulses were interrupted (Figure 5D). Recovery time for GABA inhibition was generally less than one half that required for NA.

Analysis of depressant antagonists on cerebellar Purkinje neurones

The effects of lengthening a single agonist pulse interval detailed above become important in the design of experiments to test depressant antagonists. If pulses of agonist are interrupted during the time when a putative antagonist produces depression of neuronal firing, an artifactual blockade, due to the interruption, might be observed. By using computer-generated drug response histograms, however, the agonist effects can be evaluated even during severe depression of spontaneous discharge. Several calcium antagonists, manganese, cobalt and verapamil, previously reported to be cortical adrenoceptor antagonists (Yarbrough, Lake & Phillis, 1974; Phillis & Limacher, 1974), were tested as examples of antagonists with direct depressant effects.

Figure 5 shows an experiment in which manganese was tested on cerebellar Purkinje cells using alternate NA and GABA pulses. Agonist response histograms were used to evaluate possible antagonism of the inhibitions of NA and GABA during manganese iontophoresis (Figure 5A). The histograms (Figure 5B) reveal that consistent inhibitions were produced by GABA and NA throughout the manganese application, despite the fact that iontophoresis of manganese usually produced slowing of discharge from 20 spikes per second to less than 1 per second and converted the firing pattern to irregular bursts of 5-15 spikes. Inhibitory responses to agonists during this period of direct depression are ordinarily impossible to evaluate using only ratemeter analysis (Figure 5A). Even upon cessation of manganese applications, when spontaneous rate and pattern returned to control levels, NA and GABA inhibitions remained unaffected. Not only was NA inhibition not antagonized by manganese (58 and 67% inhibitions versus control of 44%), it exhibited the same continual increase in efficiency shown in normal prolonged periods of repeated testing (Figure 4). Reapplication of manganese at a higher dose (Figure 5C) again produced no antagonism of either agonist. On the other hand, had both agonist pulses been interrupted for the duration of manganese iontophoresis (Figure 5D), an apparent blockage of both agonists would have resulted; in fact, NA efficacy was still only about 50% recovered eight pulses after the interruption. Figure 6 shows similar results in another trial of manganese on a different cell. Computer generated histogram analysis of the period of direct depression shows no antagonism of GABA or NA. Manganese, cobalt and verapamil were similarly tested on a total of six cerebellar Purkinje cells;

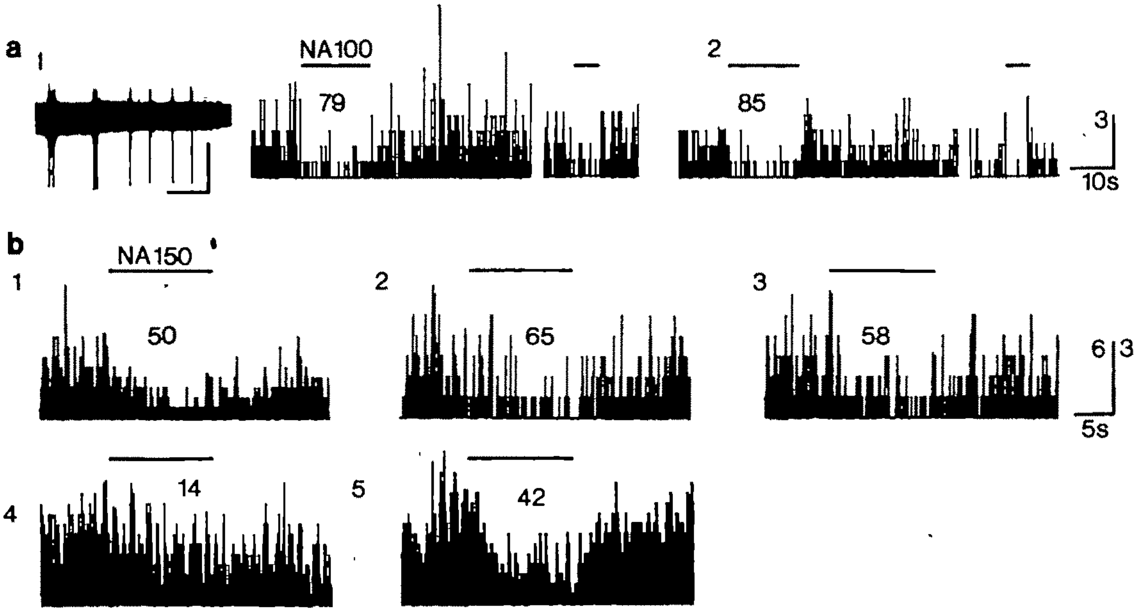

Figure 7 Effects of iontophoresis of cobalt (a) and verapamil (b) on NA-induced inhibition of cortical neurones. Two different neurones were studied in (a) and (b). A1. Control records showing action potentials (left), histogram (middle) and ratemeter (right). (a2). During 10 nA of Co iontophoresis, despite the large reduction in discharge rate shown in the ratemeter record, there is little change in the NA-induced inhibition in either the histogram (left) or the ratemeter (right). Calibration bars at right refer to (a1) and (a2) histograms only. Action potential calibration bar = 400 μV, 500 ms. (b) Effects of verapamil, 1. Control, 2. During 200 nA of verapamil. This was accompanied by a decrease in discharge rate to 10% of control, 3. Immediately after Verapamil, 4. Effects of 6 min interruption of the NA pulse, 5. Recovery of NA-induced inhibition 10 min after restoration of regular NA pulses. Horizontal calibration bar in (b3) refers to all (b) histograms Vertical bar = 6 counts per address for b1, 4, 5 and 3 counts per address for b2, 3.

partial antagonism of NA was found in only one case, with manganese (Table I).

Analysis of cerebral cortical neurones

Many cerebral cortical neurones in anaesthetized animals fire in slow, irregular discharge patterns (Figure 7A-1). The drug response histogram, by summing many agonist responses, effectively randomizes the bursts and pauses and allows quantitative determination of drug effect. Initial investigations (Yarbrough *et al.*, 1974) with manganese, cobalt and verapamil were done on cerebral cortical neurones. These agents were

Table 1 Summary of noradrenaline antagonists

	Cobalt, Manganese, Verapamil		*β-adrenoceptor antagonists**	
	Block	*No block*	*Block*	*No block*
Cerebellar Purkinje cells	1	5	18	5
Cerebral Cortical Neurones	1	7	4	0

Blockade is arbitrarily defined as a reproducible reduction of greater than 40% of NA effect. However, the block of NA by cobalt and manganese seen with 2 cells was, in fact, only partial antagonism, since the blockade was less than 40% in several of the trials.

* Data on cerebellar cells (with sotalol) from Hoffer *et al.* (1971), and Woodward, Hoffer & Altman (1974).

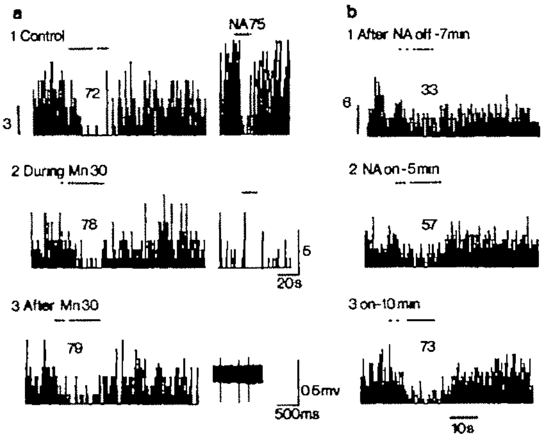

Figure 8 Effects of manganese (a) and pulse interruption (b) on NA-induced inhibitions of a cortical neuron. The same neuron was studied for both (a) and (b). (a1) Control response is shown with both histogram (left) and ratemeter (right). (a2) During iontophoresis of manganese (30 nA), NA-induced inhibition is intact as shown by the histogram (left), despite a large reduction in spontaneous discharge shown on the ratemeter (right). (a3) Histogram immediately after manganese shows NA inhibition (left). Action potential record of this cell is shown on right. (b1) Effects of a 7 min NA pulse interruption. Note large decrement in inhibitory response. (b2-3) Gradual recovery of inhibitory response over the next 10 min of continued pulsing. Histogram vertical calibrations are shown in (a1) and (b1). Note that (b) histograms are half the vertical gain of (a) histograms. Histogram time calibration shown in (b3). Ratemeter and specimen record calibrations are to the right of the lines in (a2) and (a3), respectively.

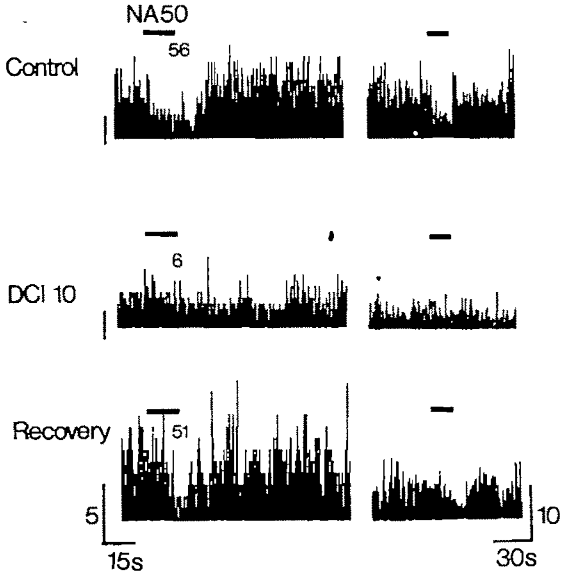

Figure 9 Blockade of NA-induced inhibition of a cerebral cortical neuron by Dichloroisoproterenol (DCI). Ratemeter records of typical single drug responses are shown the right of each NA histogram. 1. Control. 2. During 10 nA of DCI. This dose of DCI slowed discharge rate to a level similar to that seen with cobalt and manganese, as shown by the ratemeter.

studied in the cortex using the histogram technique.

As in cerebellum, cobalt, verapamil and manganese caused marked depression of spontaneous activity in cerebral cortex (ratemeter records in Figures 7A, 8A). Noradrenaline inhibitions, tested repeatedly during application of these substances and analyzed by the histograms, were not usually antagonized. Examples are shown for cobalt (Figure 7A), verapamil (Figure 7B1-3) and manganese (Figure 8). Note that during manganese iontophoresis, for instance, spontaneous rate fell to less than one spike per second (Figure 8A-2), while NA inhibition remained at 78%, compared to a control of 72%. If NA applications were interrupted for a time equal to the length of manganese application, however, inhibition fell to 33% and did not recover for 10 min (Figure 8B). The same effect is shown in Figure 7B-4, 5. Manganese, cobalt and verapamil were tested on eight cortical neurones. A slight antagonism (20%) of NA inhibition was seen in only one case, with cobalt (Table I).

For purposes of comparison, DCI was investigated as an example of β-adrenoceptor antagonist with depressant side effects. Iontophoresis of DCI nearly abolished spontaneous discharge of cerebral cortical neurones, an effect comparable to that seen with manganese, cobalt or verapamil. However, NA-induced depression was also blocked. Inhibition was reduced from 55% to 6% in one example (Figure 9). Recovery to 51% was seen 3 min later. DCI blockade of NA inhibitions was observed in all four cells studied (Table I).

Discussion

The strategy of the automated drug delivery and drug response analysis system presented here is an approach to the problems encountered in studying adrenoceptor inhibition. This system has made possible the demonstration of effective inhibition in cells firing too slowly, either inherently or due to depressant antagonists, for critical ratemeter analysis.

Problems with direct depression have been circumvented in previous studies by the selection of antagonists with which such an effect is minimal. Thus, sotalol (Hoffer *et al.*, 1971) rather than propranolol was employed to demonstrate β-adrenoceptor blockade on cerebellar Purkinje cells. Although sotalol is a less potent antagonist, it is also a less potent anaesthetic. Similarly, fluphenazine (Freedman & Hoffer, 1975) was chosen rather than chlorpromazine to demonstrate phenothiazine antagonism of NA, because of a more favourable ratio of antagonistic to direct depressant properties. The use of computer-generated histograms should now widen the range of drugs which can be tested, as well as making more quantitative analyses possible.

Expression of the extent of inhibition as a percentage change in firing rate, rather than as an absolute difference, would appear to be the most useful formulation, since, as shown in the examples, percentage inhibition remains relatively constant despite wide variations in spontaneous rate. Other parameters have been used as appropriate measures of inhibition. Hill & Simmonds (1973) calculated T_{50}, the time required to reach one-half of maximal inhibition, as a measure of GABA effects. Such a calculation could also be made with the histograms. The log dose-response curves suggested by Kelly & Renaud (1973), also for use with amino acids, are yet another possible response index. We have found, however, that several pulses of NA may be required to reach a new steady state of inhibition whenever ejection currents are changed. The reason for this phenomenon may be that a lower ejection current results in fewer residual drug ions in the tip and, therefore, less inhibition when the next pulse is applied. Thus, a given current might result in less or more inhibition depending on whether the preceding ejection current was lower or higher, an effect similar to that of pulse interruption. These phenomena do not seen to be as marked with GABA and other amino acid transmitters and, therefore, the log dose-response technique might be more applicable to those putative transmitter substances.

Irregularly firing cells, such as cortical or caudate neurones, have previously been studied under the influence of brief pulses of excitatory amino acids (Yarbrough *et al.*, 1974; Feltz & de Champlain, 1972) or low steady levels of these drugs (Nelson, Hoffer, Chu & Bloom, 1973; Connor, 1970). In view of potential interactions which might occur at sites other than the postsynaptic receptor (Nicoll, 1971), caution must be used in interpreting such data. It would be important to determine, for example, if the percentage of inhibition produced by NA and GABA is as independent of the level of amino acid-induced discharge as has been shown here for spontaneous activity. Other more physiological approaches have included stimulation of specific synaptic or sensory input to produce neuronal discharge (Foote, Freedman & Oliver, 1974; Kelly & Renaud, 1973). Such experiments provide, of course, a more detailed view of the effects of inhibition on both spontaneous and evoked activity.

The apparent decrease in inhibition resulting from interruption of a series of ejection pulses can be predicted from the studies of Hill & Simmonds (1973) and Bradshaw *et al.* (1973b) on the relationship between holding currents and drug release. Using radio-labelled NA, Bradshaw *et al.* (1973a) found that application of weak holding currents for as short a period as 10 min could reduce release *in vitro* for over 30 minutes. They proposed that the holding currents lower drug concentration in the tip as ions from the bath are drawn into the pipette. Our experiments using alternate pulses of NA from two barrels also support the hypothesis that reductions in inhibition seen after interruption of periodic drug applications is due to a diminution of iontophoretic release. One would predict that drug pulses from the barrel used without interruption would also diminish in efficacy if one of a variety of biological factors, such as saturation of reuptake sites, were of major importance.

Although we have studied only fourteen neurones in the cerebral and cerebellar cortices, our preliminary results revealed little adrenoceptor antagonism with manganese, cobalt and verapamil. This contrasts with results of Yarbrough *et al.* (1974) who described these agents as being potent antagonists. Their protocol called for interruption of NA testing for periods of up to 5 min while cells were exposed to, and recovered from, the direct depressive effects of the antagonists. Our results indicate that NA pulses would be expected to be only partly effective for several minutes after such an interruption. Tested with uninterrupted periodic pulsing from the NA pipette, these substances are generally not antagonistic even when causing a marked direct depression of the neurone. By contrast, the β-adrenoceptor antagonist DCI, which also has depressant and agonist activity, abolished NA inhibitions in cortex, confirming the report of Stone (1973).

The assumed adrenoceptor antagonism by manganese, cobalt and verapamil was cited as a main support for a theory of calcium mediation of NA effects (Yarbrough *et al.*, 1974), as opposed to mediation by adenosine 3′,5′-monophosphate (cyclic AMP; Siggins, Hoffer & Bloom, 1971). Our failure to find such antagonism by no means argues against a role for calcium. Calcium has been shown biochemically to be necessary for NA modulation of adenyl cyclase (Rasmussen, 1970) and may be important in other NA or cyclic AMP dependent processes as well. Furthermore, lanthanum, another putative calcium antagonist, effectively antagonizes NA inhibition of cerebellar Purkinje cells (Freedman & Hoffer, unpublished results). However, interpretation of such effects remains complex since lanthanum also blocks the increase in cyclic AMP produced in myometrium by isoprenaline, while incubation in calcium-free medium potentiates the isoprenaline-induced increase (Kroeger & Marshall, 1974). Hence, lanthanum may modify cyclic-AMP mediated events either directly or through calcium sensitive mechanisms which are as yet unresolved.

Techniques based on the peri-stimulus time histogram have been used in this paper to quantify the efficacy of putative adrenoceptor antagonists. With these techniques it has been possible to avoid potential artifacts resulting from inconsistent iontophoretic release of agonists and problems with irregularly discharging cells and depressant antagonists. The employment of these tactics should, thus, enhance the usefulness of microiontophoresis in the analysis of adrenergic mechanisms.

The authors are grateful for the critical comments of Drs F.E. Bloom, G.R. Siggins and F. Weight, as well as for the assistance of Mrs A. Smith and Mr H. Poole. This work was supported in part by NSF Grant GB-43301. DJW was supported by NIH TIST Award NS11030.

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*(Received December 18, 1974,
Revised March 4, 1975)*

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EFFECTS OF NARCOTIC ANALGESIC DRUGS ON THE CYCLIC ADENOSINE 3′,5′-MONOPHOSPHATE-ADENYLATE CYCLASE SYSTEM IN RAT BRAIN

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1 The concentrations of cyclic adenosine 3′,5′-monophosphate (cyclic AMP), measured in discrete brain areas removed from rats killed by microwave irradiation, rose transiently in most areas after the administration of morphine. The most pronounced changes, however, were found 2 h after doses of either 10 or 60 mg/kg morphine when cyclic AMP levels declined significantly in the hypothalamus, medulla and cerebellum. In morphine-tolerant rat brains there were no decreases in cyclic AMP levels.

2 Basal adenylate cyclase activity in crude nerve-ending fractions from discrete areas of rat brain was unaffected by the addition of active analgesic agonists, antagonists or inactive isomers to the assay medium *in vitro*, except for a nonspecific inhibition at drug concentrations of 1 mM.

3 The acute administration of morphine or levorphanol, but not dextrorphan produced transient increases in basal cyclase activity of crude nerve-ending preparations from midbrain and striatum. In morphine-tolerant rats, these changes in basal adenylate cyclase activity were no longer seen.

Introduction

The acute administration of narcotic analgesic drugs to experimental animals produces dose-related biphasic effects on the levels of biogenic amines in brain (Way & Shen, 1971) and on the rates of their biosynthesis (Clouet & Ratner, 1970; Smith, Sheldon, Bednarczyk & Villarreal, 1972). The effects of narcotic drugs on the cyclic nucleotides in brain that are involved in adrenergic transmission have been examined indirectly, for the most part; i.e. the administration of cyclic adenosine 3′,5′-monophosphate (cyclic AMP) intracerebrally has been shown to reverse the depressant effect of morphine (Ho, Loh & Way, 1972). The fact that adenylate cyclase activity in the central nervous system is stimulated by catecholamines, a phenomenon which has been demonstrated both *in vitro* (Kakiuchi & Rall, 1968a and b; Daly, Huang & Shimizu, 1972; Kebabian, Petzold & Greengard, 1972) and *in vivo* (Burkard, 1972; Garelis & Neff, 1974), suggested that a study of the effects of morphine and other narcotic analgesics on the rates of biosynthesis and the concentrations of cyclic AMP in homogenates of discrete brain areas, and of the activity of adenylate cyclase in crude nerve-ending preparations from the same areas, after treatment of rats with narcotic analgesic drugs and related compounds might be rewarding.

Methods

Male Wistar rats weighing between 100 and 130 g were injected subcutaneously with 10 or 60 mg/kg morphine hydrochloride, 15 mg/kg levorphanol or dextrorphan tartrate (Hoffmann-LaRoche) or equivalent volumes of 0.9% w/v NaCl solution (saline) for acute experiments. Morphine pellets containing 75 mg of morphine base, prepared as described by Way, Loh & Shen (1969), were implanted subcutaneously to induce tolerance. Tolerance was also induced by subcutaneous injections twice daily for 10 days of morphine in increasing doses from 10 to 100 mg/kg per injection. In one experiment, four groups of rats were injected subcutaneously with saline or morphine (60 mg/kg) and two groups with naloxone (1 mg/kg) 10 min later. All rats were killed 2 h after the initial injection. (−) and (+)-Methadone and naloxone hydrochloride were obtained from Eli Lilly and Endo Laboratories, respectively.

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Assay for cyclic AMP

After appropriate treatment, rats were placed in a styrofoam box within a microwave oven (Litton Model 70/75) and irradiated for 38 s (the time found to result in highest cyclic AMP concentrations in brain), a procedure similar to that described by Schmidt, Hopkins, Schmidt & Robison (1972). The rats were decapitated, the heads cooled on ice and the brains were removed and dissected into six areas: cortex, medulla, cerebellum, hypothalamus, striatum and midbrain, as described by Glowinski & Iversen (1966). The concentrations of cyclic AMP were measured by a protein-binding assay similar to that described by Gilman (1970), except that the binding protein was isolated from rabbit skeletal muscle instead of beef muscle, and 100 mM sodium acetate buffer, pH 4.5 was used for the binding reaction (Miki, Yamamoto & Iwatsubo, 1972). Brain protein was measured after precipitation with 5% trichloroacetic acid (TCA) by the method of Lowry, Rosebrough, Farr & Randall, 1951.

Adenylate cyclase assay

After decapitation, the brains were removed and dissected into six areas as described above. The tissues were homogenized in 0.32 M sucrose in sufficient volumes to make tissue suspensions of 30 mg wet weight/ml, and the crude mitochondrial-synaptosomal fraction isolated by the established sucrose density gradient centrifugation procedure (Chakrin & Whittaker, 1969). For *in vitro* incubations the crude mitochondrial-synaptosomal fraction was recentrifuged in order to isolate a nerve-ending fraction. The tissue pellets were suspended in water to make final protein concentrations of approximately 2 μg/μl.

In the cyclase assay, 50 μl of tissue suspension was added to 150 μl of a mixture containing 0.5 to 1 μCi [^{32}P]-adenosine 5′-triphosphate (0.1 μmol, New England Nuclear Corp.), $MgSO_4$ (1 μmol), theophylline (2 μmol), cyclic AMP (0.2 μmol), phosphoenolpyruvate (1 μmol), pyruvate kinase (1.9 i.u. with 2.2 μmol ammonium sulphate, Sigma Chemical Co.) and tris (hydroxymethyl)-methylammonium hydrochloride (Tris) (8 μmol), pH 7.5 (and drugs, when tested *in vitro*) and incubated at 30°C for 5 minutes. In preliminary experiments, shown in Table 2, the ATP-generating system (phosphoenolpyruvate and pyruvate kinase) was omitted and caffeine (1.3 μmol) was used as a phosphodiesterase inhibitor instead of theophylline. This assay was terminated after 10 min at 30°C. In each assay, the enzyme reaction was stopped by heating the assay vessels to 100°C for 2 minutes. One ml of the Tris buffer containing 10,000 to 20,000 ct/min of [^{3}H]-cyclic AMP (New England Nuclear Corp.) was added before centrifugation in order to calculate recovery of cyclic AMP in each sample. The cyclic AMP fraction was separated from other components on a column of neutral aluminium oxide as described by White & Zenser (1971) and twice clarified by $ZnSO_4$-$Ba(OH)_2$ precipitation, as described by Krishna, Weiss & Brodie (1968). The ^{3}H and ^{32}P radioactivity in the supernatant fraction was measured in a Nuclear-Chicago scintillation spectrometer. In control experiments, (1) [^{3}H]-cyclic AMP was added to the aluminium column and carried through the procedure with 60-70% recovery; and (2) phosphodiesterase (from beef heart, Sigma Chemical Co.) was added to the assay with the elimination of radioactivity in the final supernatant fraction. Under the experimental conditions described, enzyme activity was proportional to tissue concentration and time in both assays.

Statistics

The data were examined for significance of differences by Student's *t* test.

Results

Concentrations of cyclic AMP after acute treatment with morphine

After the administration of morphine (60 mg/kg, s.c.), transient rises in cyclic AMP concentrations were found in all areas except hypothalamus and medulla (Figure 1). In the latter regions, both 10 and 60 mg/kg doses of morphine produced significant decreases in cyclic AMP concentrations within 1 or 2 hours. In the cerebellum, both doses of morphine produced biphasic changes in cyclic AMP concentrations which were maximal at 15 or 30 min and returned to control or lower levels 2 h after morphine administration.

Concentrations of cyclic AMP in brains of tolerant animals

Rats made morphine-tolerant by 10 days of morphine injections were killed 2 h after the last injection. In brain regions of these rats, the concentrations of cyclic AMP were unaltered from control values in all areas except cortex and striatum (Table 1), in which there were significant increases. Compared to cyclic AMP concentrations attained 2 h after the initial injection of 60 mg/kg, the levels of the nucleotide were higher in all areas

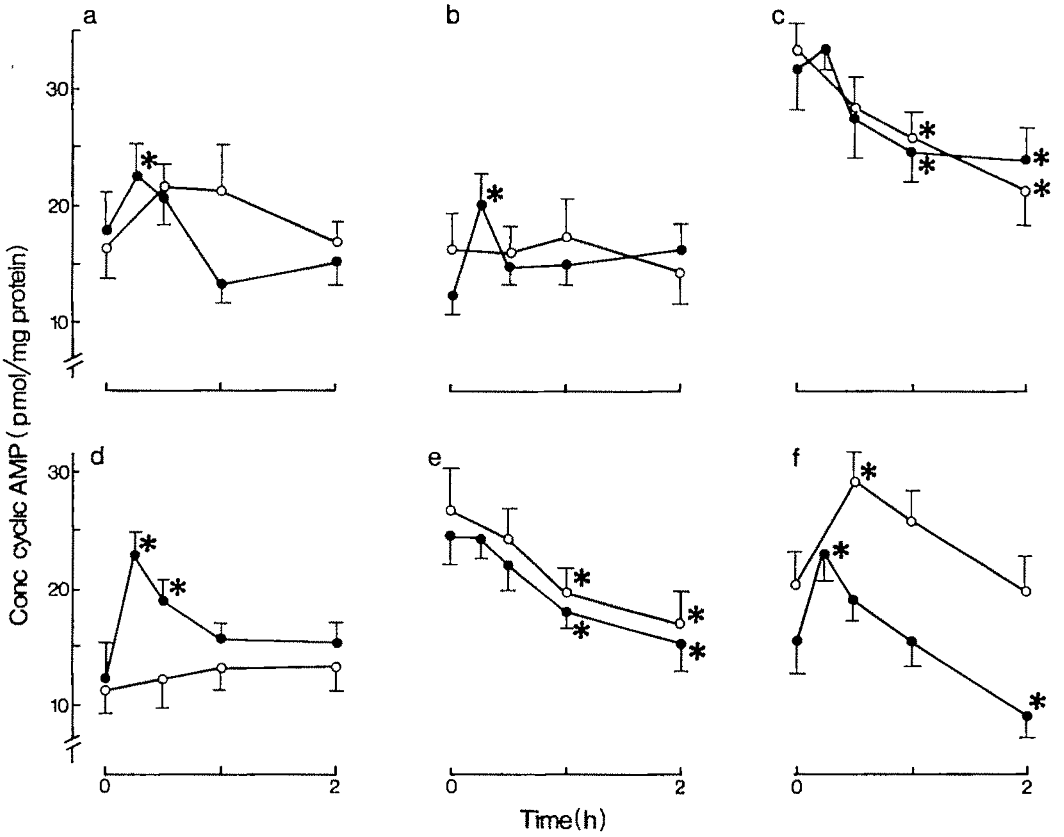

Figure 1 Concentration of cyclic AMP in areas of rat brain after the acute administration of morphine. (a) midbrain, (b) striatum, (c) medulla, (d) cortex, (e) hypothalamus, (f) cerebellum. (○) Morphine 10 mg/kg s.c.; (●) 60 mg/kg s.c. Morphine was administered to rats that were subsequently killed at intervals up to 2 hours. The zero-time values are from untreated rats. Each value represents the mean from four rats. Vertical lines show s.e. mean. *Values differ significantly from appropriate zero-time control values ($P < 0.05$).

Table 1 Concentrations of cyclic AMP in brain areas from morphine-tolerant rats

Brain area	*Control* (pmol cyclic AMP/mg protein)	*Morphine-tolerant*† (pmol cyclic AMP/mg protein)
Midbrain	26.5 ± 0.7	27.1 ± 0.6
Striatum	12.8 ± 0.3	*21.4 ± 0.3
Medulla	26.9 ± 0.3	27.1 ± 0.3
Cortex	9.2 ± 0.1	*16.6 ± 0.4
Hypothalamus	21.6 ± 0.7	24.6 ± 0.8
Cerebellum	26.0 ± 0.3	24.2 ± 0.1

† Rats were made tolerant by injections twice daily with morphine in increasing doses from 10 to 100 mg/kg for 10 days, and were killed 2 h after the last injection. Control animals received saline in equivalent amounts. Each value represents the mean ± s.e. mean of four animals.

* Significantly different from control values at $P < 0.001$.

in the tolerant animals. Thus, the depleting effect of morphine administered acutely was not found in tolerant animals.

Effects of opioids on adenylate cyclase activity in vitro

The effects on basal adenylate cyclase activity of the addition of morphine, levorphanol, and its less active isomer, dextrorphan, (−)- and (+)-methadone and the antagonist, naloxone, are shown in Table 2. At 1 mM concentration, levorphanol and dextrorphan reduced cyclase activity by about 50%, and the other opioids had lesser inhibitory activity. At lower drug concentrations, there was either no effect or slight inhibition of adenylate cyclase activity in the ruptured nerve-ending fractions.

Effects of acute treatment with opioids on adenylate cyclase activity

After the 60 mg/kg dose of morphine adenylate cyclase activity was increased in the crude nerve-ending preparations from midbrain, striatum and medulla, and decreased in cerebellum (Figure 2). After levorphanol 15 mg/kg, adenylate cyclase activity was increased in midbrain and striatum and decreased in cerebellum, while the same dose of dextrorphan produced no significant changes in cyclase activity. A dose of naloxone (1 mg/kg) that blocked the analgesic and hypothermic responses to morphine (60 mg/kg) did not block the increases in adenylate cyclase activity in midbrain and striatum 2 h after the morphine injection (Table 3). There was a trend toward increased adenylate cyclase activity in the medulla and a trend toward decreased adenylate cyclase activity in the cerebellum, although the differences were not statistically significant in this experiment.

Effects of tolerance on adenylate cyclase activity

Rats were made morphine-tolerant by the implantation of one morphine pellet subcutaneously on day 1, two pellets on day 5, followed by twice daily injections of morphine (100 mg/kg) on days 9 and 10. Tolerant rats in one group (B) were killed 18 h after the last injection, while those in another group (C) received another injection 2 h before they were killed. The untreated rats (group A) were sham-operated for pellet implantation on days 1 and 5. There were no changes in the basal activity of adenylate cyclase in any region in group B (Figure 3). However, in group C, there were significant increases above control values in several areas of brain, namely midbrain, cerebellum and cortex (Figure 3).

Discussion

The transient changes in the levels of cyclic AMP

Table 2 Effect of narcotic analgesics and related compounds on adenylate cyclase activity in nerve-endings *in vitro*

Adenylate cyclase activity (pmol cyclic AMP min^{-1} mg protein^{-1})	*Midbrain*	*Striatum*	*Medulla*	*Cortex*	*Hypothalamus*	*Cerebellum*
	218 ± 87	*430 ± 49*	*193 ± 22*	*392 ± 47*	*380 ± 36*	*617 ± 75*
Drugs (mM)			*(% of control activity)†*			
Morphine						
0.1	99	96	102	91	105	115
1.0	98	90	99	70*	89*	108
Levorphanol						
0.1	81*	101	91	90	72*	104
1.0	37*	49*	47*	56*	42*	59*
Dextrorphan						
0.1	84*	106	87*	98	82*	100
1.0	37*	57*	39*	48*	49*	50*
(—)-Methadone						
0.1	103	93	103	93	96	88*
1.0	78*	68*	95	57*	89*	74*
(+)-Methadone						
0.1	92	84*	95	71*	100	93
1.0	85*	73*	85*	71*	96	73*
Naloxone						
0.1	87*	110	91	113	104	108
1.0	81*	105	97	94	81*	108

† The drugs were added to purified ruptured nerve-ending preparations at the final concentrations specified, and adenylate cyclase activity was measured (by a different assay from that used otherwise throughout the paper, see Methods sections for details). Adenylate cyclase activity in the presence of drugs is expressed as % of the control values shown in the top line and is the average of three observations.

* Values are different from control values at $P < 0.05$.

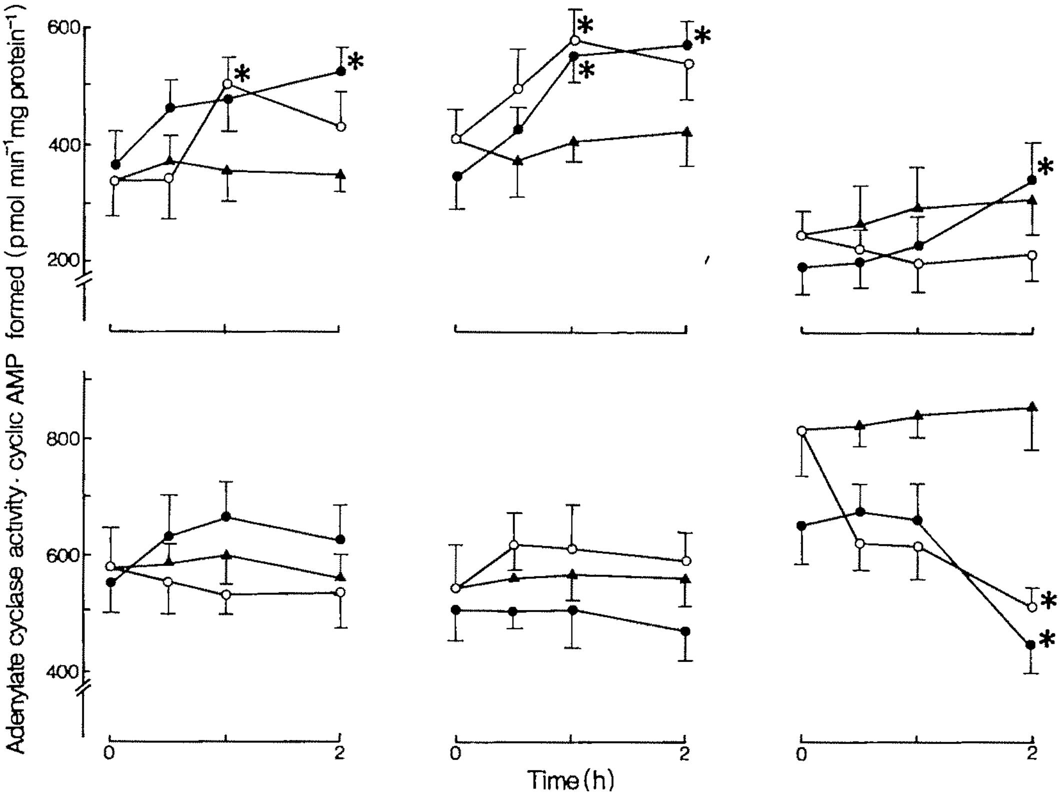

Figure 2 Adenylate cyclase activity (expressed as cyclic AMP formed) in crude nerve-ending preparations from rat brain areas after the acute administration of morphine, levorphanol or dextrorphan: (a) midbrain, (b) striatum, (c) medulla, (d) cortex, (e) hypothalamus, (f) cerebellum. (●) Morphine 60 mg/kg; (○) levorphanol 15 mg/kg; (▲) dextrorphan 15 mg/kg. Each value represents the mean from three rats. Vertical lines show s.e. mean. *Values are significantly different from respective zero-time control values ($P < 0.05$).

Table 3 Effect of naloxone and/or morphine administration on adenylate cyclase activity in nerve-endings

Treatment	*Midbrain*	*Striatum*	*Medulla*	*Cortex*	*Hypothalamus*	*Cerebellum*
			(% of control activity)†			
Saline	100 ± 7	100 ± 10	100 ± 13	100 ± 9	100 ± 13	100 ± 11
Morphine	*149 ± 8	*148 ± 9	129 ± 12	100 ± 10	100 ± 11	79 ± 8
Saline + naloxone	106 ± 7	98 ± 2	103 ± 17	98 ± 8	99 ± 9	98 ± 7
Morphine + naloxone	**183 ± 8	**167 ± 12	*143 ± 9	103 ± 10	102 ± 12	84 ± 11

† Morphine (60 mg/kg, s.c.) or saline was injected into four groups of rats each. Naloxone (1 mg/kg, s.c.) was injected 10 min later into the last two groups, and all rats were killed 2 h after the initial injection. Each value represents the mean ± s.e. mean % of adenylate cyclase activity in nerve-ending preparations from the same brain regions in saline-treated control rats.

* $P < 0.05$; ** $P < 0.02$, (significance of differences between morphine-treated and appropriate control groups). There were no differences between morphine and morphine plus naloxone values.

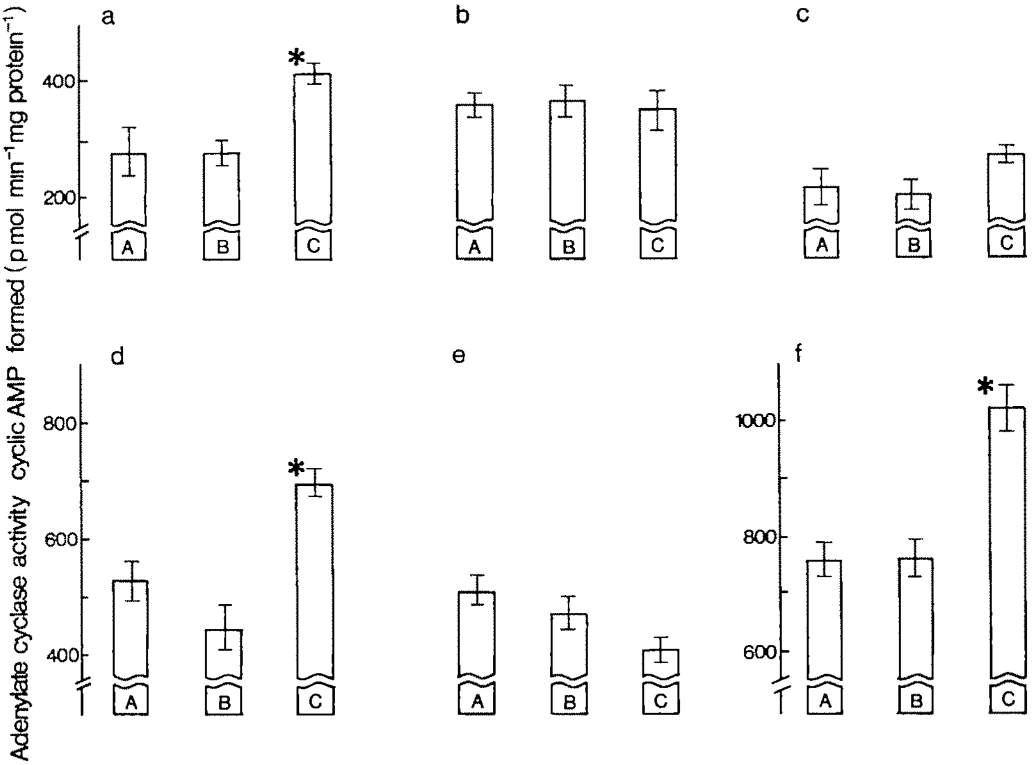

Figure 3 Adenylate cyclase activity in crude nerve-ending preparations from brain areas from morphine-tolerant rats (a) midbrain, (b) striatum, (c) medulla, (d) cortex, (e) hypothalamus, (f) cerebellum. Columns A: Sham-operated control rats. B: Rats treated chronically with morphine as described in the text and killed 18 h after the last injection. C: Rats treated chronically with morphine as in group B, but with an additional injection of morphine 100 mg/kg 2 h before they were killed. Each column represents the mean result from four rats. Vertical lines show s.e. mean. *Values differ significanty from sham-operated controls ($P < 0.05$).

in some brain areas after the administration of morphine are not incompatible with a 'second messenger' role for cyclic AMP in the responses to morphine. However, the changes were not related temporally to basal adenylate cyclase activity in nerve-ending preparations from the same brain areas, with the possible exception of the cerebellum in the second hour after acute morphine. The time course of alterations of cyclic AMP concentrations was also not related temporally to transient changes in the concentrations of catecholamines in the same brain areas after acute morphine treatment, which we described earlier (Johnson, Ratner, Gold & Clouet, 1974). However, a close correlation between the rate of catecholamine release and cyclic AMP concentrations in a brain area should not be anticipated because the cyclic AMP synthesized by neurotransmitter-sensitive adenylate in membranes may be a small part of the total pool of cyclic AMP measured in tissue homogenates.

The addition of narcotic agonists, the antagonist naloxone, or relatively inactive stereoisomers of agonists, at drug concentrations below 1 mM to purified nerve-ending membrane preparations *in vitro* caused little change in adenylate cyclase activity. At 1 mM concentration, most of the narcotic drugs and related compounds inhibited the enzyme activity to some extent, with highest inhibition by the isomers, levorphanol and dextrorphan, showing that there was no stereospecificity in the inhibition of cyclase activity.

Adenylate cyclase activity in the crude mitochondrial-nerve ending fraction was higher in some areas (midbrain, medulla and striatum) and lower in cerebellum after acute morphine administration at a dose of 60 mg/kg. A lower dose (25 mg/kg) of morphine did not cause any change in adenylate cyclase activity of crude mitochondrial fractions of mouse cerebral cortex (Naito & Kuriyama, 1973). Similarly, a dose of 15 mg/kg morphine did not alter either the activities of adenylate cyclase or concentrations of cyclic AMP in homogenate of rat cortex, cerebellum and thalamus or hypothalamus 1 h after the injection (Singhal, Kacew & Lafreniere, 1973). Thus, it seems that only a high dose of morphine, which caused profound sedation and catalepsy, could produce significant changes in basal adenylate cyclase activity.

When the effect of morphine administration on adenylate cyclase activity in the isolated crude mitochondrial fraction was compared with that of levorphanol at an equianalgesic dose to morphine and of dextrorphan at the same dose, only levorphanol produced some effects similar to those of morphine on adenylate cyclase activity while dextrorphan had no effect on cyclase activity. Thus, the effect of narcotic drugs *in vivo* on the adenylate cyclase activity seemed to show stereospecificity. Pharmacological specificity, however, was not demonstrated, since a dose of naloxone that blocked the pharmacological responses to morphine did not prevent the increases in cyclase activity induced by morphine. It may be noted that, although there were no statistically significant differences in samples from morphine or morphine plus naloxone treated rats, the trend was toward reversing the decrease in cerebellar activity and enhancing the increase in cyclase activity in other areas. Should this trend be confirmed in further studies, one possible interpretation would be that naloxone, when administered after morphine as in our experimental conditions, was acting as an antagonist in the cerebellum and as an agonist in the medulla and striatum.

In tolerant animals, basal adenylate cyclase activities were the same as in naïve animals in all brain areas, unless an additional injection of morphine (100 mg/kg) was administered 2 h before the rats were killed. The concentrations of cyclic AMP in tolerant rats killed 2 h after the last injection of morphine were higher than those in animals treated acutely, an indication either of tolerance in the response of the cyclic AMP-adenylate cyclase system, or of tolerance in interacting systems in the brain. That the largest changes in the concentration of cyclic AMP and in adenylate cyclase activity produced by the acute administration of morphine are found in the cerebellum, a tissue in which stereospecific opiate binding in *in vitro* systems is low (Kuhar, Pert & Snyder, 1973; Hiller, Pearson & Simon, 1973), suggests that the responses to opiates in the adenylate cyclase system are not a direct consequence of opiate-receptor binding *in situ*, but rather secondary responses (possibly transmitted by the noradrenergic pathway from the locus coeruleus).

This investigation was supported in part by a research grant from the National Institute on Drug Abuse, Rockville, Maryland (DA-00087). A preliminary report was presented at the FASEB meeting in April, 1973. The authors wish to thank Eli Lilly and Endo Laboratories for gifts of (−)- and (+)-methadone and of naloxone hydrochloride respectively.

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(Received November 18, 1974.
Revised May 7, 1975.)

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VOLUME 54, 1975

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